No. 2014-1547

UNITED STATES COURT OF APPEALS FOR THE FEDERAL CIRCUIT

THE TRUSTEES OF COLUMBIA UNIVERSITY
IN THE CITY OF NEW YORK,

Appellant,

v.

ILLUMINA, INC.,

Appellee.

On Appeal from the United States Patent and Trademark Office,
Patent Trial and Appeal Board, No. IPR2012-00006

NONCONFIDENTIAL BRIEF FOR APPELLANT

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September 29, 2014

AMENDED CERTIFICATE OF INTEREST

Counsel for Appellant certify the following:

1. The full name of every party or amicus represented in this appeal is:

The Trustees of Columbia University in the City of New York.

2. The names of the real parties in interest represented in this appeal are:

Not applicable.

3. The names of all parent corporations and any publicly held companies that own 10 percent of the party represented are:

None.

4. The names of all law firms and the partners or associates who appeared for the party or amicus now represented by me in the trial court or agency or are expected to appear in this court are:

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STATEMENT OF RELATED CASES

This is an appeal from *inter partes* review ("IPR") of U.S. Patent No. 7,713,698 ("the '698 patent") before the Patent and Trademark Office's Patent Trial and Appeal Board ("the Board"), with Illumina, Inc. as petitioner and The Trustees of Columbia University in the City of New York ("Columbia") as patent owner. No other appeal from the IPR was previously before this Court or any other court.

Two related appeals are also pending in this Court from IPRs between Columbia, as patent owner/appellant, and Illumina, as petitioner/appellee: No. 2014-1548, an appeal from IPR2012-00007 of U.S. Patent No. 7,790,869; and No. 2014-1550, an appeal from IPR2013-00011 of U.S. Patent No. 8,088,575. This Court previously ordered that the three appeals be considered companion cases, but denied Columbia's motion to consolidate them. Dkt. Nos. 13, 16.

Columbia sued Illumina for infringement of five DNA sequencing patents, including the three at issue in these appeals, in March 2012. *Trustees of Columbia Univ. v. Illumina, Inc.*, No. 12-cv-376 (D. Del.). That litigation is stayed, but for limited fact discovery, pending the outcome of these and other IPR proceedings. *See* Dkt. No. 89, *Columbia Univ.*, No. 12-cv-376 (D. Del. Apr. 1, 2013).

JURISDICTION

The Board issued its final written decision on March 6, 2014. A1. Columbia noticed a timely appeal on May 2, 2014. A2454; *see* 35 U.S.C. § 142; 37 C.F.R. § 90.3(a). This Court has jurisdiction under 35 U.S.C. § 141(c).

ISSUES ON APPEAL

1. Whether the Board violated the statutory framework for IPRs by, among other defects, shifting the burden of proof to Columbia and making numerous findings for which Illumina produced no supporting evidence, as though the Board were functioning as an examiner rather than an impartial arbiter.

2. Whether the Board erred in finding original claims 1-7, 11-12, 14-15, and 17 obvious where no substantial evidence supports its conclusions regarding motivations to combine the prior art or reasonable expectations of success.

3. Whether the Board improperly disregarded compelling objective indicia of nonobviousness.

STATEMENT

I. BACKGROUND

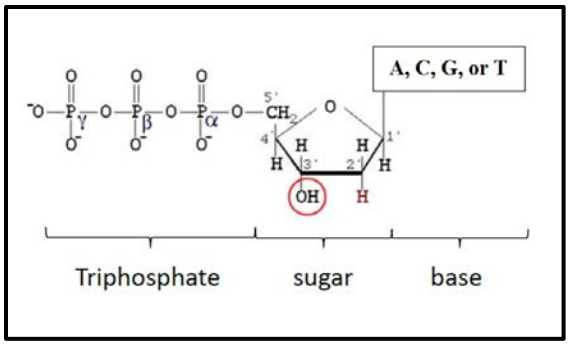
A. DNA Nucleotides

The patents in these appeals concern methods of sequencing deoxyribonucleic acid (DNA) using modified versions of naturally occurring nucleotides (or "nucleotide analogues"). DNA encodes the genetic information of

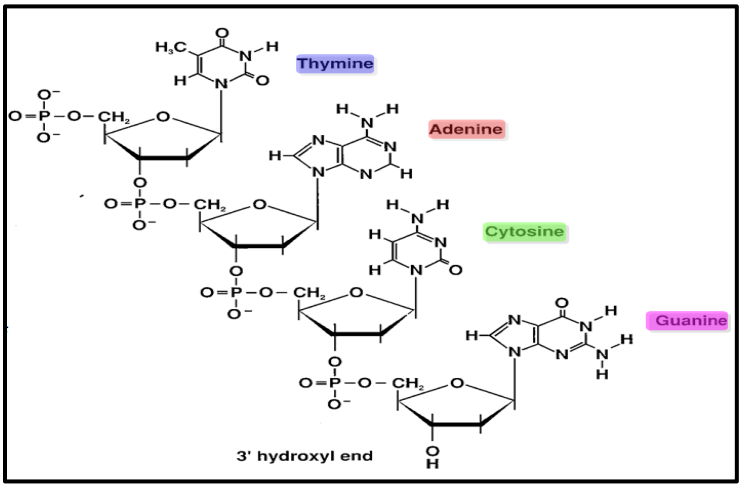
living organisms. DNA consists of smaller building blocks called nucleotides; the sequence of the nucleotides determines hereditary traits in living organisms. DNA sequencing—*i.e.*, determining the order of the nucleotides in a DNA strand—is of enormous importance for a wide variety of applications in medicine, biotechnology, and other fields. For example, by sequencing the DNA of individuals with a particular disease, medical researchers may learn the genetic basis for it and may design therapies specifically targeted to it.

A nucleotide consists of a sugar, a base, and one or more phosphate groups, as shown below. In a naturally occurring nucleotide, the base is either adenine, guanine, cytosine, or thymine—A, G, C, or T. Adenine and guanine are known as "purine" bases, while cytosine and thymine are "pyrimidine" bases. The sugar in the nucleotide contains five carbon atoms, conventionally numbered 1′ through 5′. When the nucleotide is found in isolation, a hydroxyl group (OH) is attached at the 3′ position and is referred to as the 3′-OH group (circled below). The nucleotide depicted below has a triphosphate group and is called a deoxyribonucleoside triphosphate or "dNTP" (or "dATP," "dGTP," etc. to indicate a specific base).[1]

[1] A "nucleoside," unlike a nucleotide, contains only a sugar and a base. Nucleotides can be equivalently referred to as nucleosides with added phosphate groups (hence, "deoxyribonucleoside triphosphate").

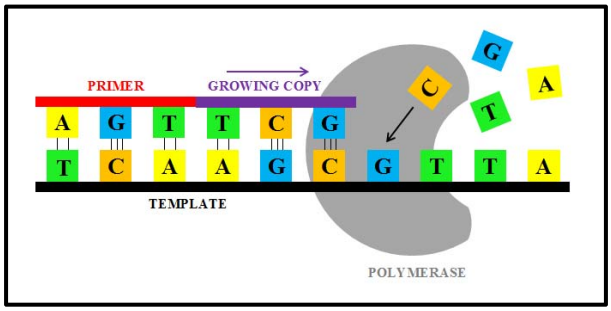


DNA consists of a chain of nucleotides, held together by bonds between the phosphate group of one nucleotide and the sugar of the next nucleotide in the chain. Specifically, the phosphate group of one nucleotide binds with the 3′-OH of the previous nucleotide:

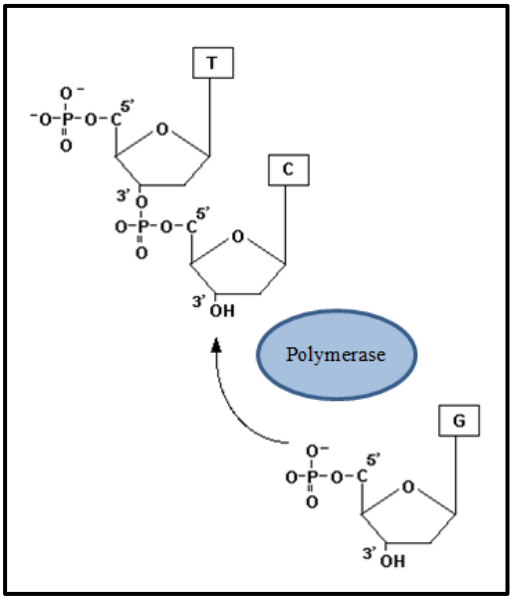


DNA occurs naturally in a double helix structure, which is two chains of nucleotides held together by hydrogen bonds between complementary base-pairs: C to G and A to T. When DNA is copied—for example, during DNA sequencing—the two strands of this helix are first unwound and separated. Each chain of nucleotides can then serve as a "template" to be copied. A shorter chain of nucleotides, called a "primer," binds to the starting point of the sequence to be

copied. An enzyme called a polymerase then assembles a new chain of nucleotides with bases complementary to those of the template being copied in the pairings noted above (A-T and C-G).



The polymerase works by facilitating the formation of a bond between the phosphate group of a free nucleotide and the 3′-OH group of the last nucleotide in the growing chain. For this reaction to take place, the last nucleotide already in the chain must have a 3′-OH group available for binding to the phosphate group of the incoming nucleotide, as depicted below.



B. Sanger Sequencing

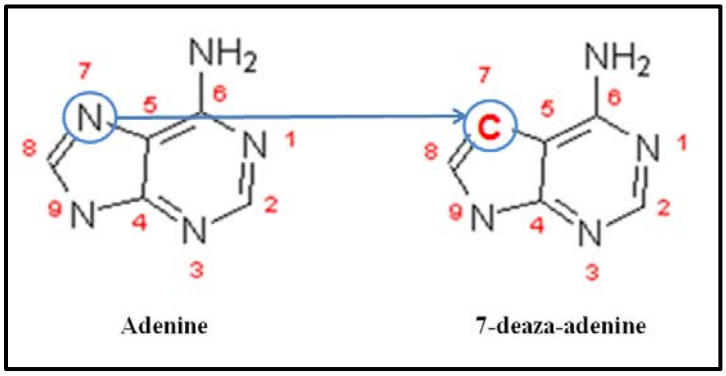
In the 1970s, Sanger invented a sequencing method that remained dominant for decades. A3788-3789; A3773-3777. Sanger sequencing relied on modified nucleotides called *dideoxy*nucleotides ("ddNTPs"), which had only a hydrogen atom at the 3′ position of the sugar rather than the OH group found in dNTPs. A3773. If a ddNTP was incorporated into a nucleotide chain, "the chain [could not] be extended further" because there was no 3′-OH group to which the polymerase could bind another nucleotide. *Id.* This chain termination was irreversible; once a ddNTP was incorporated, the DNA synthesis stopped permanently. A3280:15-3282:10; A3789-3790.

Sanger sequencing also relied on detectable labels. Originally, these labels were radioactive. A3773. Prober, Trainor, and colleagues improved Sanger sequencing by using fluorescent labels attached to the chain-terminating ddNTPs, with a unique label for each of the four bases. A3789; *see* Prober et al., *A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides*, 238 Science 336 (1987) ("Prober") (A3062-3067).

To sequence DNA using this version of Sanger sequencing, DNA templates were mixed with a polymerase, a primer, dNTPs, and a small amount of fluorescently labeled ddNTPs. A3063. The result was a series of fragments that all began the same way but terminated at different points, wherever the polymerase

happened to incorporate a chain-terminating ddNTP rather than a dNTP. The fragments were then sorted by size (a proxy for length) using a technique called electrophoresis. The last nucleotide in each fragment—the chain-terminating ddNTP—was identified by "reading" its fluorescent label. The complete DNA sequence was then deduced from the relative length of each fragment and each fragment's chain-terminating ddNTP. *See* A3788-3789.

Another improvement to the original Sanger method involved the use of deazapurines to address problems associated with electrophoresis. A3789-3791. A deazapurine is a purine base in which a nitrogen atom has been replaced with a carbon atom; the term "7-deazapurine" indicates that the change is at the 7 position. A65; A3316:20-3317:2.



In Sanger sequencing, deazapurines were used in the irreversible chain-terminating ddNTPs because labels attached to the 7-position carbon of the deazapurine were more stable than labels attached to the 7-position nitrogen of a natural purine; stability of the label was important because the label had to remain

attached during the harsh conditions associated with electrophoresis. A3063; A3789-3790; *see* A3065 (describing electrophoresis). Deazaguanosine triphosphates ("deaza-dGTPs") were also used to avoid problems encountered in the electrophoresis step of Sanger sequencing when sequencing guanine-cytosine-rich ("G-C rich") regions of DNA. A3790-3791; A3850. These deaza-dGTPs were not labeled and were not chain-terminating nucleotides.

C. The Prior Art Problem

In the late 1980s, researchers conceived of an alternative to Sanger sequencing called sequencing by synthesis ("SBS"). A3791-3792. Sanger sequencing depended on electrophoresis, which was a "bottleneck" to sequencing massive numbers of DNA templates. A3791; A3458:24-3459:9; A132 (2:3-6). Higher-throughput methods were needed to sequence entire genomes, which could be billions of nucleotides long. A3739:12-18.

SBS dispensed with electrophoresis and sought to detect the sequence of a nucleotide chain directly as nucleotides were added to it. A3791-3792. One proposed, but ultimately unsuccessful, SBS method used nucleotides with labeled, removable "caps," or "blocking groups," attached to the 3′-OH group on the sugar portion of the nucleotide. A3792-3793. When a polymerase incorporated such a nucleotide into a growing chain, the synthesis would stop because the 3′-OH group—where the polymerase would otherwise join the phosphate group of the

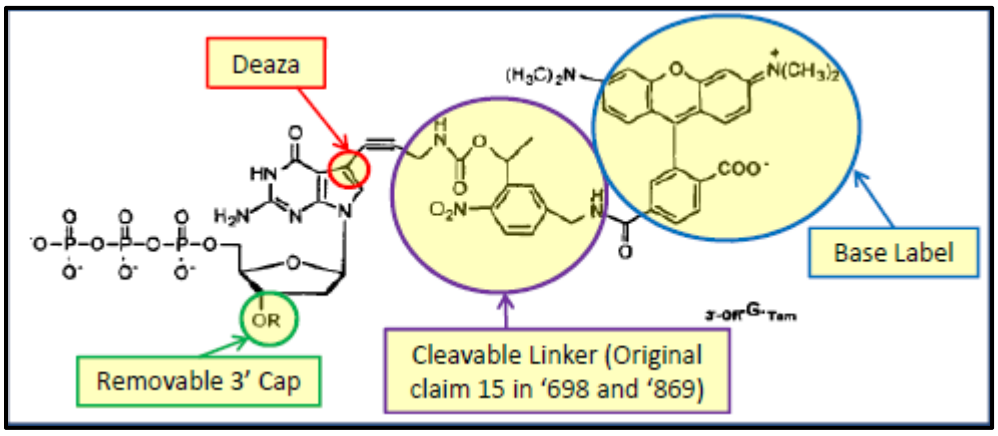
next nucleotide—was blocked by the labeled cap. Unlike the *irreversible* chain-terminating ddNTPs in Sanger sequencing, these chain-terminating dNTPs were *reversible*. After the label was detected, the labeled cap on the 3′-OH group could be removed, allowing the polymerase to add another nucleotide to the chain. A3792-3793; A132 (2:33-37).

That, at least, was the theory. This early SBS method yielded only "[l]imited success for the incorporation of the 3′-modified nucleotide by DNA polymerase." A132 (2:37-39). As Dr. Jingyue Ju of Columbia would later recognize, the reason for this limited success was that 3′-OH caps bearing a bulky label were not spatially compatible with the polymerase. Specifically, Dr. Ju's insight was that "the 3′-position on the deoxyribose," where the labeled cap was attached, "is very close to the … active site of the polymerase, and the polymerase is therefore sensitive to modification in this area" of the nucleotide. *Id.* (2:39-43).

Thus, despite its limitations and despite years of efforts to develop an SBS alternative for high-throughput sequencing, Sanger sequencing remained the mainstay of DNA sequencing from the 1970s to the early 2000s. A3788. As Illumina's witness stated, Sanger sequencing had a virtual "monopoly" on sequencing for a remarkable 25 years. A3466:6-13; *see* A3466:14-16; A3467:22-3468:6. "[N]o complete success of using [an SBS] system to unambiguously sequence DNA ha[d] been reported" as of October 2000. A3732:22-3733:6.

D. Dr. Ju's Breakthrough

Dr. Ju and his colleagues at Columbia identified the solution that had eluded others for successful SBS. Dr. Ju realized that a labeled cap at the 3′-OH position was too bulky to be "accepted by the polymerase." A4229:11-19. That spatial insight led him to conceive and synthesize a nucleotide with an alternate arrangement of features. *Id.* Dr. Ju's SBS method uses nucleotides that have an unlabeled cap on the 3′-OH group; a separate, unique label attached to the *base*, rather than to the 3′-OH cap; a cleavable linker between the label and the base; and, when the base is a purine, a deazapurine in place of a natural purine. A3803-3804; A3806; A132 (2:57-65). This configuration is shown in figure 7 of the '698 patent (reproduced with annotations):



To sequence DNA using Dr. Ju's method, a DNA template is immobilized on a solid surface, such as a glass chip. A142 (21:40-46). A primer hybridized to the DNA template provides a substrate (a starting point) for a polymerase to add one of Dr. Ju's reversibly 3′-OH capped, base-labeled nucleotides. *Id.* (21:46-47).

Because the polymerase adds nucleotides to a growing copy starting from the primer, the newly synthesized strand is called a "primer extension strand." *E.g.*, A133 (4:53-54). Synthesis terminates when the polymerase incorporates one of Dr. Ju's nucleotides because the cap on the 3′-OH group prevents incorporation of another nucleotide. A135 (8:19-20); A142 (21:52-56). The label attached to the base of the chain-terminating nucleotide is then detected. A135 (8:30-33). Each of the four bases has a unique label. A142 (21:49-51). After detection, the 3′-OH cap and the base-label are both cleaved. A135 (8:39-45). The polymerase reaction can then resume, adding another base-labeled, 3′-OH capped nucleotide.

Unlike Sanger sequencing, Dr. Ju's SBS method permits rapid sequencing of many different DNA templates at the same time. A137 (11:58-64); *see* A3874-3875. Indeed, thousands of different DNA templates may be immobilized to different locations on the solid surface in the first step of Dr. Ju's method. A142 (22:16-22). The base-labels of the chain-terminating nucleotides on each of these templates are "read" in parallel. *Id.* (21:60-22:15). A solid surface containing high-density groupings of different DNA templates for sequencing is called a "microarray." A3286:21-25; A3507:3-14; A3508:5-3509:2.

Dr. Ju's innovation received widespread praise after he published his results in 2006. A3876-3878; *see* A3925-3930 (Dr. Ju's published article); A3953 (peer review describing it as "a novel approach" that "clearly establishes a unique and

robust strategy" that "can be extended to develop a very high throughput DNA sequencing … system that can be used routinely for a wide range of applications"). Dr. Ju's SBS method produced unexpectedly better sequencing results than the closest comparable method commercially available at the time of the invention, called pyrosequencing. A3872-3874.[2] It has now been used to sequence entire genomes at a speed and efficiency unknown before his invention. A3872; A3874-3875.

II. ILLUMINA'S RESPONSE TO DR. JU'S INVENTION

A. Copying

Manteia, a company whose intellectual property was later acquired by Illumina's predecessor-in-interest Solexa, copied Dr. Ju's invention soon after learning of it. In a May 2003 presentation, Manteia reproduced the '698 patent's figure 1—which had been published in related applications (A3893)—and repeated Dr. Ju's observation that the region surrounding the 3′ position is "very crowded" compared with positions on the base. A3969; *see* A3892-3893. In the same presentation, Manteia cited Dr. Ju's publication for the suggestion to use a "small 3′-OH" blocking group, specifying the same chemical structures as the '698 patent. A3970; *see* A3894; A133 (3:28-35).

[2] Pyrosequencing is an SBS method that relies not on nucleotide labeling but rather on detecting phosphate groups released when a nucleotide is incorporated into the growing DNA chain during the polymerase reaction. A3792.

[redacted]

B. Attempted Licensing

Illumina also sought to license Dr. Ju's technology, but lost out to an earlier bidder. In June 2005 internal discussions about Dr. Ju's invention, Illumina's executives recognized that Illumina did not "have a play" in SBS, which they believed would "be the 'next big' technology in genomics." A3993. Illumina's principal scientist for genomics, Dr. Kevin Gunderson, observed that Illumina was "missing" two "key technological components" for SBS—including "reversible terminators with a cleavable dye," of the sort Dr. Ju invented—and suggested that

Illumina "approach Columbia University regarding … licensing." *Id.*; *see id.* ("Professor Jingyue Ju purportedly has solved the reversible terminator cleavable dye label issue."). Dr. David Barker, Illumina's vice president and chief science officer, responded that he "know[s] [Dr. Ju] very well," and that "what he says [about his invention] will be true." A3992.

In December 2005, Gunderson wrote Barker again about high-throughput sequencing. Noting that Illumina was still refining its "'sequencing' approach" and "need[ed] to strengthen [its] proposal in this area," Gunderson asked: "Is there a possibility of collaborating with Jing Ju from Columbia University on his reversible terminators? Can we license this technology?" A3995. Barker reported that Dr. Ju "is amenable to collaborating with us, but we will need to get some help quickly from business development as two other groups are trying to license the technology from Columbia." A3996. Barker also reported that Dr. Ju was "ahead" of Solexa in developing nucleotide analogues. *Id.* Illumina's co-founder, John Stuelpnagel, instructed Barker to "initiate discussions with Columbia and [Dr. Ju]," and to "think hard about how to reward [Dr. Ju] in this transaction." A3997.

Columbia ultimately granted an exclusive license to a startup company, IBS, with which Dr. Ju had been collaborating. When Illumina's executives heard that such a deal was imminent, they sought to "convince[] [Columbia] that it would be in their best interest to go with a more proven company." A3999; *see also* A4004-

4011 (emails describing licensing efforts). Columbia, however, went through with its plans.

Illumina subsequently acquired Solexa (and thus Manteia's technology) and achieved considerable commercial success in the SBS market using sequencing methods with the features claimed by Dr. Ju. Nevertheless, Illumina continued its licensing efforts. In early 2008, Illumina asked Columbia what "intellectual property rights Columbia University might be able to grant Illumina in a potential collaboration between Dr. Ju/Columbia and Illumina," notwithstanding Columbia's exclusive license to IBS. A4014. Illumina also explored acquiring IBS or gaining access to its intellectual property. A4017-4030. Central to these discussions was IBS's "Columbia University license." A4024. In 2012, IBS was instead acquired by a different company—[redacted]. A4031.

C. Commercial Success

By 2013, [redacted] in the next-generation (*i.e.*, post-Sanger) sequencing market consisted of Illumina products that, when used to sequence DNA, embody the claimed features of the '698 patent—[redacted]. A4041-4042; A3880-3885. From 2006 through the first quarter of 2013, Illumina earned [redacted]

in revenue from such products (nearly all after it acquired Solexa), including operating profits of [redacted]. A4039-4041. The market dominance achieved by Illumina's embodying products has likely grown since then: [redacted]. A4047-4048.

Illumina's own scientists attributed its success to use of nucleotides with the features claimed in the '698 patent: "[H]aving a very small 3′-block and leaving the [label] on the base is the reason our SBS works so well." A35.

III. PRIOR PROCEEDINGS

In March 2012, Columbia sued Illumina for infringing the patents at issue in these appeals. Dkt. No. 1, *Trustees of Columbia Univ. v. Illumina, Inc.*, No. 12-cv-376 (D. Del. Mar. 26, 2012). In September 2012, Illumina petitioned for IPR of the '698 patent. A2000. The Board authorized review of independent claims 1 and 11 and dependent claims 2-7, 12, 14-15, and 17 as obvious in view of Tsien et al., WO 91/06678 (May 16, 1991) ("Tsien") (A3000-3061) and Prober; the same claims as obvious in view of Tsien and Seela, U.S. Patent No. 4,804,748 (Feb. 14, 1989) ("Seela") (A3155-3158); and claims 5 and 12 as obvious in view of Prober, Tsien, and Rabani et al., WO 96/27025 (Sept. 6, 1996) ("Rabani") (A3095-3154). A91-92.

A. Representative Claims

Claim 1 recites:

> A method of determining the identity of a nucleotide analogue incorporated into a nucleic acid primer extension strand, comprising:
>
> a) contacting a nucleic acid template attached to a solid surface with a nucleic acid primer which hybridizes to the template;
>
> b) simultaneously contacting the product of step a) with a polymerase and four nucleotide analogues which are either (i) aA, aC, aG, and aT, or (ii) aA, aC, aG, and aU, so as to incorporate one of the nucleotide analogues onto the nucleic acid primer and form a nucleic acid primer extension strand, wherein each nucleotide analogue within (i) or (ii) comprises **a base labeled with a unique label** and contains **a removable chemical moiety capping the 3'-OH group of the sugar** of the nucleotide analogue, and wherein at least one of the four nucleotide analogues within (i) or (ii) is **deaza-substituted**; and c) detecting the unique label of the incorporated nucleotide analogue, so as to thereby determine the identity of the nucleotide analogue incorporated into the nucleic acid primer extension strand.

A149 (35:1-23) (emphases added). Claims 2-4, 6-7, 14, and 17 all depend directly or indirectly from claim 1. Claim 5 recites the "method of claim 1, wherein in step a) a plurality of different nucleic acid templates are attached to the solid surface." *Id.* (35:34-36). Claim 15 recites the method of claim 1, where the labels are attached to the nucleotide "via **a cleavable linker**." *Id.* (36:44-46) (emphasis added).

Claim 11 is a product claim, reciting:

> **A plurality of nucleic acid templates** immobilized on a solid surface, wherein a nucleic acid primer is hybridized to such nucleic acid templates each such nucleic acid primer comprising a labeled

> incorporated nucleotide analogue, at least one of which is **deaza-substituted**, wherein each labeled nucleotide analogue comprises **a base labeled with a unique label** and contains **a removable chemical moiety capping the 3'-OH group** of the sugar of the nucleotide analogue.

A149 (36:24-31) (emphases added). Claim 12 depends from claim 11, specifying that the plurality of nucleic acid templates "are present in a microarray." *Id.* (36:32-33).

Columbia moved to amend the challenged claims during the IPR. The proposed amendments would have effectively added the cleavable-linker limitation of original claim 15 to all the challenged method claims. A2215-2216; A2228-2231. Columbia also moved to amend claim 11 (as proposed claim 25) to clarify that it requires "[a] plurality of **different** nucleic acid templates." A2216 (emphasis added).

B. The Prior Art

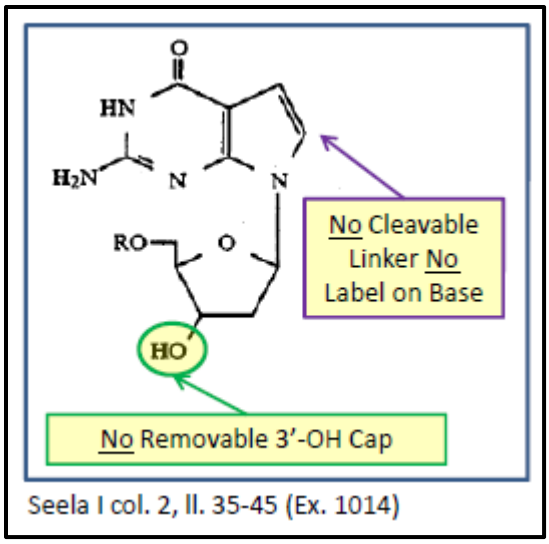
1. Prober

Prober—co-authored by Columbia's expert, Dr. George Trainor—teaches an improvement to Sanger sequencing using fluorescent labels. A3062; A3398:15-16. Prober discusses deazapurines for use in the Sanger method, but only for purposes inapplicable in SBS. First, Prober teaches that attaching a label to a deaza-substituted ddNTP "facilitate[s] stable linker" attachment of the label to the base. A3063. In the Sanger method, unlike in Dr. Ju's SBS method, the label must

remain permanently attached to the base during the harsh conditions of electrophoresis; the label is never cleaved, and, indeed, the linkers described by Prober "cannot be cleaved under any conditions that would not destroy the nucleotide." A3834; A4665:2-4666:3. Second, Prober teaches that using unlabeled, non-chain-terminating deaza-dGTPs solves resolution problems in the Sanger method related to the use of electrophoresis, which is not used in SBS. A3067.

2. Seela

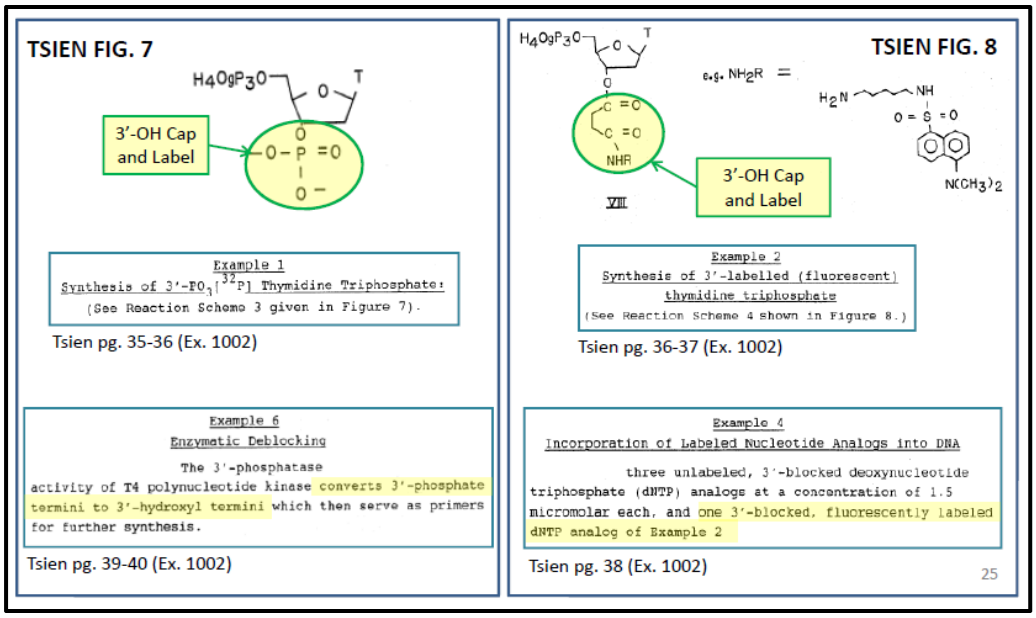
Seela, like Prober, teaches the use of deaza-substituted nucleotides in Sanger sequencing. A3157 (4:4-10). Seela explains that the deaza-substitution eliminates "secondary" interactions between C and G nucleotides, "result[ing] in a substantially better gel-electrophoretic separation of guanine-cytosine-rich sequence fragments." *Id.* (4:24-30). SBS methods do not use electrophoresis. A3791-3792; *see supra* pp.8-9. Seela does not disclose either base-labels or removable 3′-OH-caps. A3833-3834; A3850. For example, the "general formula" Seela discloses for deaza-substituted nucleotides is unlabeled and uncapped. A3156 (col. 2) (annotated below).



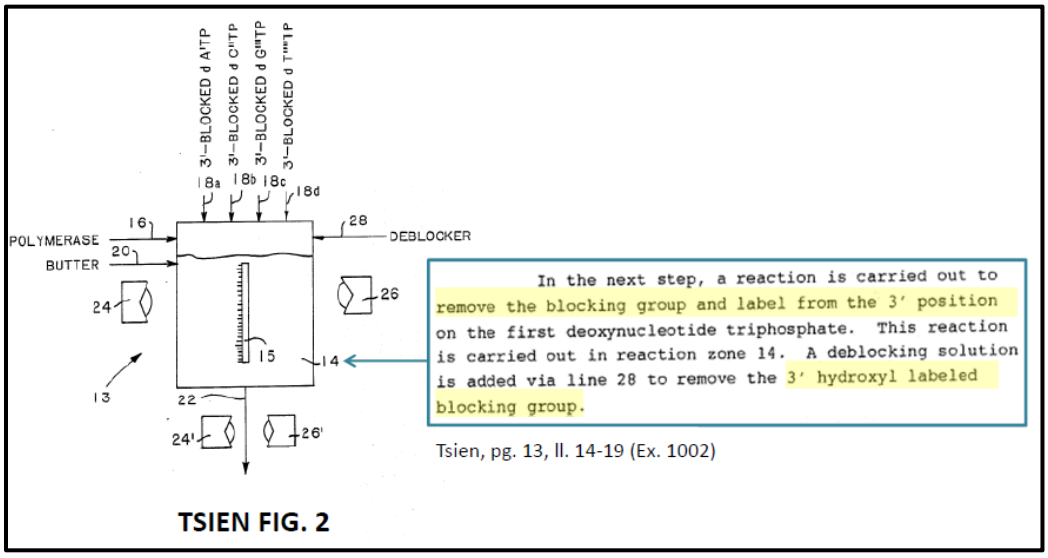
Seela I col. 2, ll. 35-45 (Ex. 1014)

3. Tsien

Tsien, a 1991 patent application, describes an early attempt to design methods for SBS. A3007:28-33. Tsien hypothesizes that "many different configurations" of labeled, 3′-OH capped nucleotides could be used in its proposed methods, including ones in which the label "is incorporated within the blocking group [*i.e.*, attached to the 3′-OH cap] or is otherwise incorporated in a way which allows it to be removed between each addition." A3008:19; A3015:14-22; A3818. Although Tsien broadly discusses other configurations (*see infra* pp.21-22), the only molecular-structure diagrams of 3′-OH-capped nucleotides in the reference show labels attached to the 3′-OH caps, not the bases. *See* A3057-3058; A3819-3820; A4830 (demonstrative reproduced below).



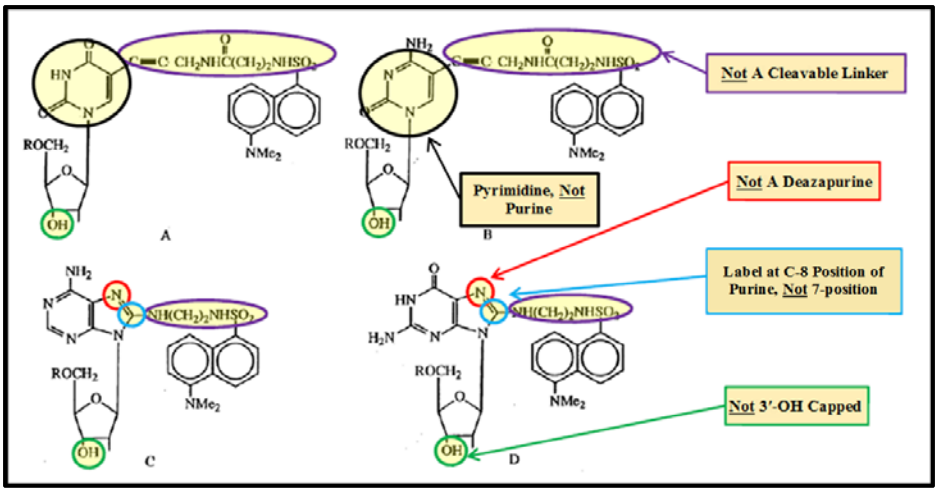
Tsien's preferred embodiment uses a "3′[-OH] labeled blocking group" and a single reaction to "remove the blocking group and label from the 3′ position." A3014:14-16; A4829 (demonstrative reproduced below).



After describing in detail "approaches to labeling focus[ed] on incorporating the label into the 3′-[OH] blocking group," Tsien suggests that "a number of alternatives" might be possible, including "a label … coupled to a remote position

such as the base." A3028:33-3029:2; *see also* A3029:5-6 (suggesting a "fluorescent tag attached to the base moiety"). In the following paragraph, Tsien also suggests that "[i]n another type of remote labeling," the label can be attached "through a spacer or tether," which "can be cleavable if desired." A3029:19-22.

Tsien does not discuss deazapurines. Tsien does refer to Prober, but never for labeling a purine, let alone a deazapurine. First, Tsien notes that Prober had shown that *irreversible chain-terminating* ddNTPs could be incorporated by a polymerase. A3029:16-18. Second, Tsien states that a "number of approaches are possible to" labeling *pyrimidine* bases, not *purines*, including "[o]ne … based on" Prober. A3030:10-14. For *purines*, by contrast, Tsien cites a different reference and teaches that attaching a label to the natural (non-deaza) 8-position carbon of the purine is "ideal." A3030:3-4. Tsien then presents four nucleotide diagrams, none showing a deazapurine (or a cleavable linker or 3′-OH cap). A3829-3832; A3031 (annotated below).



4. Rabani

Rabani is a 1996 PCT application drawn to systems for "massively parallel examination" of complex molecules, including but not limited to systems for the parallel examination of labeled DNA nucleotides. A3097:4-6. Rabani states that this proposed method for parallel examination of complex molecules is subject to the "constraints entailed by the particular instrumentation" used for detection of the molecules. A3107:4-7. Rabani then suggests several possible detection instruments, including "multiple probes (i.e., in arrays with parallel detection provided)." A3107:12-13. Rabani does not, however, describe a microarray of nucleic acids. A3840-3841.

C. The Trial

After the Board granted Illumina's petition, Columbia moved to amend the challenged claims. A2215-2216. The amendment would have effectively added the cleavable linker limitation of original claim 15 to all the other challenged method claims and would have clarified that claim 11 requires a "plurality of **different** nucleic acid templates." *See supra* p.18.[3]

[3] The Board directed Columbia to file its motion to amend at the same time as its merits response. A2100. Accordingly, Columbia's response was addressed to the proposed amended claims. The Board ultimately denied the motion to amend, though it appeared to agree with Columbia that a skilled artisan would understand claim 11 as requiring a plurality of *different* templates. A43.

Columbia's expert, Dr. Trainor, testified that, prior to Dr. Ju's invention, a skilled artisan considering a potential SBS method would have viewed a synthetic nucleotide with a labeled 3′-OH cap as more likely to be incorporated than alternatives because of its structural similarity to a natural nucleotide. A3818-3819; A4667:21-4668:13. That was why Tsien focused on labeling the 3′-OH cap. A3852; A4422:11-4425:19. Dr. Trainor also explained that a skilled artisan at the time would have seen labeling the cap as "synthetically attractive" because it required modifying fewer parts of the nucleotide, and so required fewer steps to synthesize. A4668:14-4669:5.

Dr. Trainor further testified that, even if Tsien had provided a motivation to make a nucleotide with a 3′-OH cap and a label attached to the base for use in a sequencing method, there would have been no reason to expect success at doing so. Tsien does not teach the necessary chemistry, leaving the challenge of "creat[ing] completely new chemical procedures." A3835; A3852. Unlike mechanical parts that may easily be moved, taking the base-labels from Prober's ddNTPs and adding them to Tsien's 3′-OH capped nucleotides would have required "design[ing] new chemical synthesis procedures" not taught by those two references. A3827. Seela teaches a synthesis procedure, but not for base-labeled or 3′-OH-capped nucleotides; no chemistry was available to modify the nucleotides taught in Seela

to include a base label, a cleavable linker (as required by claim 15), or a removable 3′-OH cap. A3851-3852.

Although Dr. George Weinstock testified for Illumina that "mixing together things" from the references "would actually work" (A3560:20-24), he admitted that he is not a chemist (A3266:1-5). Dr. Weinstock disclaimed any expertise on matters of synthetic nucleotide chemistry, repeatedly stating that he would need to "consult a colleague." A3389:3-8; *see* A3366:20-3367:3; A3388:25-3389:11; A3414:6-3415:2; A3495:4-10; A3498:6-3499:18; A3551:5-10; A3715:21-3716:16. Dr. Weinstock admitted that he had never done any work in connection with the synthesis of nucleotides. A3266:11-13; A3279:16-19.[4]

[4] Illumina introduced additional references following the institution of the IPR and submitted a "rebuttal" declaration from a chemist, Dr. Kevin Burgess, with its reply. A2259-2262; A2288. Columbia moved to exclude those exhibits and the related portions of Dr. Burgess's declaration as an improper attempt by Illumina to bolster its prima facie case. A2290-2294; *see Office Patent Trial Practice Guide*, 77 Fed. Reg. 48,756, 48,767 (Aug. 14, 2012). The Board dismissed Columbia's motion as moot because it held the claims unpatentable without considering the additional references or Dr. Burgess's declaration. A44-45. Because the Board did not consider this evidence, it is not part of the "information upon which the Board relied in rendering its decision" and may not be a basis for affirmance in this Court. *In re Gartside*, 203 F.3d 1305, 1314 (Fed. Cir. 2000); *see SEC v. Chenery Corp.*, 332 U.S. 194, 196 (1947); *In re Thrift*, 298 F.3d 1357, 1367 (Fed. Cir. 2002). In any event, Dr. Trainor testified that many of the additional references were not DNA sequencing art and that others *confirmed* that the preferred approach prior to Dr. Ju's invention was to label a 3′-OH cap. *See, e.g.*, A3858-3870; A4356:21-4357:1; A4369:4-4370:3; A4372:8-25; A4377:4-4380:6; A4423:2-4424:10; A4496:18-4487:19.

Dr. Weinstock was also unable to identify any motivation in these references for using a deazapurine, as in Dr. Ju's claimed method. Illumina argued that the art provided a motivation to use a deazapurine because Tsien refers to Prober, which uses deazapurines. A2032-2033. However, Tsien refers to Prober only for labeling *pyrimidines*, and to a different reference for labeling *purines*. *See supra* p.22. Furthermore, Dr. Trainor explained that the two reasons given in Prober for using deazapurines in Sanger sequencing—stable attachment of the label and improved electrophoresis for G-C rich regions—are not relevant to the claimed method. A3789-3790; A3850. The '698 patent claims a method of SBS, which does not involve electrophoresis. A3791-3792. Seela also would not have provided any motivation to use a deazapurine; the deaza-substituted nucleotides in Seela are for use in Sanger sequencing to overcome the same G-C rich region electrophoresis problem described in Prober. A3833-3834. Even Dr. Weinstock acknowledged that electrophoresis problems were not relevant to Dr. Ju's method. A3377:23-3378:14.

Dr. Trainor also testified that the asserted references would not have provided a skilled artisan with *a reasonable expectation of success in making* a 3′-OH capped nucleotide with a base-label and a deazapurine. A3826-3828; A3834-3835; A3837; A3850-3852; A3854. He further explained that the asserted references gave no reason to expect that such a cobbled-together nucleotide would

be incorporated by a polymerase into a primer extension strand—a required step of all the challenged method claims. A3837-3838; A3854-3855.

Moreover, even if an artisan made such a nucleotide, Dr. Trainor testified that it would not have been obvious in view of the asserted references to use the nucleotide in a sequencing method with multiple different DNA templates immobilized on a solid surface. A3838-3839; A3855.[5] Illumina had pointed in its petition to a passage in Tsien discussing "[a] plurality of copies of a … DNA" molecule immobilized on a surface, depicted in Tsien's figure 2. A2027; *see* A3012:34-36; A3053. Dr. Weinstock acknowledged, however, that the cited passage refers only to multiple copies of "a single" template, not multiple *different* templates. A3516:4-8; *see* A3547:3-8 (similar).

Nor would Rabani have filled this gap. Although Rabani generally describes parallel examination of "diverse" molecules (A3097:4-6), Rabani does not describe sequencing a plurality of different DNA templates in a microarray, as claimed in Dr. Ju's invention. A3840. The only purported teaching of a "microarray" cited by Illumina is an array of "detection instrumentation"—that is, an array of the

[5] Claim 5 recites using Dr. Ju's method with a "plurality of *different* nucleic acid templates" attached to a solid surface. A149 (35:34-36) (emphasis added). Claim 11 recites "[a] plurality of nucleic acid templates immobilized on a solid surface." *Id.* (36:24-25). Dr. Trainor and Dr. Weinstock both agreed that claim 11 would have been understood in context by a skilled artisan as the product of the method of claim 5, and thus as also requiring a plurality of *different* nucleic acid templates. A3816-3817; A3332:22-3333:19.

instruments to be used to examine molecules, not an array of molecules to be examined, and decidedly not an array of DNA templates. *See* A2056; A3187; A3840-3841.

Finally, Columbia introduced evidence (summarized above) of objective indicia of nonobviousness, including copying, attempted licensing, commercial success, and unexpected advantages.

D. The Final Written Decision

The Board held all of the challenged claims unpatentable. A45.

First, it held that original claims 1-7, 11-12, 14-15, and 17 were obvious in view of Tsien and Prober. A9-27.[6] Although Tsien states a preference for attaching the label to the 3′-OH cap, the Board read it as also teaching a nucleotide with a removable 3′-OH cap and a label attached to the base via a cleavable linker. *See* A14-15; A22-24. In its view, using a deazapurine with such a nucleotide would have been obvious in view of Tsien's reference to Prober. A17-20. As to Columbia's argument that the references did not teach the requisite synthetic chemistry, the Board stated that the question whether Tsien and Prober provided a reasonable expectation of success at making the claimed nucleotide had been "addressed above" in its decision (A27), although it had not been. The Board also did not address the "plurality of nucleic acids" limitation of claim 11 and stated

[6] The Board made separate substantive findings only as to claim 1 and claim 15, which requires a cleavable linker. A9; A14; A27.

that Columbia "did not separately argue claim 11" (which Columbia did, as proposed claim 25). *Id.*; *see* A2191.

Second, the Board held claims 5 and 12 obvious in view of Tsien, Prober, and Rabani. A27-28. It made no separate findings for this rejection and stated instead that it was "adopt[ing] the findings and reasoning set forth in [its] Decision on Petition." A28; *see* A89-90 (petition decision).

Third, the Board held that original claims 1-7, 11-12, 14-15, and 17 were obvious in view of Tsien and Seela. A28. It made no separate findings for this rejection either, concluding that "[t]he reason for combining the cited publications is the same as for Tsien and Prober." *Id.*

Finally, the Board discounted Columbia's objective evidence of nonobviousness. A28-39. In its view, Illumina's commercial success was due to features readily available in Tsien (A34-37), and Illumina's attempted licensing did not reflect "recognition of the merits of the claimed invention" (A38). The Board did not address Columbia's evidence of copying.

SUMMARY OF ARGUMENT

The Board's decision in this case violated the statutory framework for IPRs. Congress designed IPRs to be adjudicative, not examinational. Yet, the Board effectively treated these proceedings as an examination. It relied on "findings" from its decision to institute, even though that decision is not evidence; it relied on

its own reading of the prior art, without supporting expert testimony; and it shifted the burden of proof to Columbia on critical questions of fact.

As an initial matter, the Board failed to resolve the parties' dispute over whether a person of ordinary skill in the art would have been skilled in synthetic chemistry. Illumina's expert, Dr. Weinstock, is not a chemist and was unqualified to opine on chemistry questions central to this case.

The Board wrongly concluded that a method using nucleotides combining the features of Dr. Ju's nucleotide was obvious. Tsien provided no motivation to try to use a nucleotide with a label on the base, rather than the 3′-OH cap, and no reason to expect success in making such a nucleotide. Had an artisan made a base-labeled nucleotide, attaching the label via a cleavable linker (as required by claim 15) would not have been obvious from Tsien, who did not teach a nucleotide with both a base-label and a cleavable linker.

Combining these features with the deazapurine teachings of Prober or Seela also was not obvious. Prober and Seela taught deazapurines for Sanger sequencing, for reasons not relevant to Dr. Ju's SBS method. Neither reference provides a synthesis procedure for making 3′-OH-capped, base-labeled deaza-nucleotides.

Even if a skilled artisan had made such a nucleotide, the references gave no reason to expect that it would be incorporated by a polymerase—a critical part of

the invention and one of the most unpredictable challenges in SBS. And none of the references taught using such a nucleotide to sequence a plurality of different nucleic acid templates in a microarray.

Columbia provided powerful objective evidence of nonobviousness. Manteia, a company whose IP Illumina later acquired, copied Dr. Ju's invention shortly after learning of it—evidence the Board failed to address. Illumina itself sought to license Dr. Ju's invention, a sign of respect for the invention that the Board wrongly discounted by speculating that Illumina was seeking to license features taught by Tsien or to avoid litigation. Illumina has achieved substantial commercial success using products that embody Dr. Ju's method, a fact that the Board again wrongly attributed to individual features it perceived as available in Tsien, rather than to the claimed invention as a whole. Dr. Ju's invention was also unexpectedly better than the closest commercially available prior art.

ARGUMENT

I. STANDARDS OF REVIEW

This Court reviews the Board's legal conclusions de novo and its factual findings for substantial evidence. *Rambus Inc. v. Rea*, 731 F.3d 1248, 1251 (Fed. Cir. 2013). "'Substantial evidence is more than a mere scintilla. It means such relevant evidence as a reasonable mind might accept as adequate to support a conclusion.'" *In re Gartside*, 203 F.3d 1305, 1312 (Fed. Cir. 2000).

"Obviousness is a question of law based on underlying factual findings: (1) the scope and content of the prior art; (2) the differences between the claims and the prior art; (3) the level of ordinary skill in the art; and (4) objective indicia of nonobviousness." *InTouch Techs., Inc. v. VGo Commc'ns, Inc.*, 751 F.3d 1327, 1347 (Fed. Cir. 2014).

II. THE BOARD VIOLATED THE STATUTORY FRAMEWORK FOR *INTER PARTES* REVIEW

The Board's decision in this case cannot be reconciled with the role Congress envisioned for it in IPRs in the Leahy-Smith America Invents Act ("AIA"). Pub. L. No. 112-29, § 6, 125 Stat. 284, 299-305 (2011). The AIA converted the prior *inter partes* reexamination procedure "from an examinational to an adjudicative proceeding." H.R. Rep. No. 112-98, at 46 (2011). Senator Kyl, one of the architects of the AIA's IPR provisions, explained that this was an "important structural change" for the Board, which the PTO had sought to avoid burdening the Board with the onus of proving unpatentability. 157 Cong. Rec. S1375 (Mar. 8, 2011); *see* 157 Cong Rec. S1040-S1041 (Mar. 1, 2011) (statement of Sen. Kyl).

Under the "oppositional system" that Congress adopted (and the PTO advocated), the petitioner and patent owner "present their evidence to the PTO, which then simply decides whether the petitioner has met his burden." 154 Cong. Rec. 22,625 (2008) (statement of Sen. Kyl). For this reason, an IPR petition must

identify "with particularity … the grounds on which the challenge … is based, and the evidence that supports the grounds." 35 U.S.C. § 312(a).

Several important principles follow from Congress's conception of IPR as an "adjudicative proceeding." H.R. Rep. No. 112-98, at 46. The Board's decision to institute a review is *not* a prima facie determination of unpatentability that the patent owner must rebut, but merely a determination that "there is a reasonable likelihood that the petitioner would prevail with respect to at least 1 of the claims challenged in the petition." 35 U.S.C. § 314(a); *see* 37 C.F.R. § 42.108(c). Even after review is instituted, the petitioner always bears the burden of proving that the claims are unpatentable by a preponderance of the evidence, and the burden never shifts to the patent owner. 35 U.S.C. § 316(e); H.R. Rep. No. 112-98, at 47. If the petitioner fails to carry its burden, the challenge must fail. The Board's role is to serve as "an impartial adjudicator of an adversarial dispute between two parties," and not to scour the record to develop arguments, not made by the petitioner, for the patent owner to rebut. *Brand v. Miller*, 487 F.3d 862, 869 (Fed. Cir. 2007) (pre-AIA adversarial interference); *see* 35 U.S.C. § 312(a). As the fact-finder in an adjudicative proceeding, the Board must identify the level of ordinary skill in the pertinent art, including the qualifications of the skilled artisan, and then make its determination regarding obviousness from the perspective of a skilled artisan. *See Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966); *Daiichi Sankyo Co. v. Apotex,*

Inc. (*Daiichi I*), 501 F.3d 1254, 1256-1257 (Fed. Cir. 2007); *DyStar Textilfarben GmbH v. C.H. Patrick Co.*, 464 F.3d 1356, 1361-1363 (Fed. Cir. 2006).

The Board violated these principles here, effectively treating the proceeding as an examination. It relied in its final decision on "factual findings" that it claimed to have made in its decision to institute (A28), even though the decision to institute is not evidence and is not a determination of unpatentability. It failed to resolve a critical dispute between the parties over the level of ordinary skill in the pertinent art—specifically, whether a skilled artisan would have been skilled in both chemistry and biology, or in biology only. *Compare* A3801-3802, *with* A3165-3166. It relied on its own reading of the prior art, unsupported by expert testimony. *See, e.g.*, A10-11; A19-20; A25-26. Finally, it shifted the burden of proof to Columbia on several critical questions of fact. *See, e.g.*, A24; A28.

These legal errors, described below, pervaded the Board's decision and render its findings unsupportable.

III. THE BOARD ERRED IN CONCLUDING THAT A SKILLED ARTISAN WOULD HAVE COMBINED THE PRIOR ART TO ACHIEVE DR. JU'S INVENTION

Original claim 15 recites a method of SBS using nucleotides with "a base labeled with a unique label," a "cleavable linker" attaching the label to the base, and "a removable chemical moiety capping the 3′-OH group," where at least one of the nucleotides is "deaza-substituted." A149 (35:14-18, 36:44-46). Original claims 1-7, 14, and 17 recite a method using nucleotides with all these features,

without the cleavable-linker limitation. In finding these claims obvious, the Board made three unsupported inferential leaps: First, that a skilled artisan would have made a *base-labeled*, 3′-OH-capped nucleotide, rather than one in which the label was attached to the 3′-OH cap itself; second, as to claim 15, that a skilled artisan would have attached the label to the base by means of a *cleavable linker*; and third, that a skilled artisan would have used a *deazapurine* in place of a natural purine base.

For each inference, the Board should have considered whether a skilled artisan "'would have been motivated to combine the teachings of the prior art'" and whether the artisan "'would have had a reasonable expectation of success in doing so.'" *InTouch*, 751 F.3d at 1347; *see Otsuka Pharm. Co. v. Sandoz, Inc.*, 678 F.3d 1280, 1292 (Fed. Cir. 2012). For the motivation-to-combine analysis in particular, the Board should have considered not merely what a skilled artisan "*could*" have done, but what the skilled artisan "*would*" have done, at the time of the invention and without the benefit of hindsight. *InTouch*, 751 F.3d at 1352.

The Board did not do so. It either failed to make the required findings or based its "findings" on bare assertions unsupported by record evidence. The evidence relied on by the Board cannot support a finding of obviousness.

A. The Board Failed To Identify The Skilled Artisan

As an initial matter, the Board failed to resolve the parties' dispute regarding the qualifications of the person of ordinary skill in the art—a basic step in the obviousness analysis. *Daiichi I*, 501 F.3d at 1256-1257; *DyStar*, 464 F.3d at 1361-1362. Columbia argued that the skilled artisan would have been skilled in *both* biology and synthetic nucleotide chemistry with "a graduate degree in chemistry or chemical biology or a related discipline" and "knowledge of synthetic nucleotide chemistry." A2173-2174; A3801-3802. Columbia's witness, Dr. Trainor, met these criteria. A2174. Illumina's witness, Dr. Weinstock, did not: He lacked a degree or "formal training" in chemistry and had never "done any work in connection with the synthesis of nucleotides." A3266:1-13; *see* A3160. Dr. Weinstock was thus unqualified to opine on matters of synthetic nucleotide chemistry, as he repeatedly conceded. *See supra* p.25. Dr. Weinstock maintained, however, that the skilled artisan need only have been skilled in "molecular biology or associated sciences," not chemistry. A3166.

The Board did not resolve this disagreement, simply stating that each witness was "qualif[ied] to testify on the issues discussed in his declaration." A3. The Board's failure to resolve this disagreement was legal error. Moreover, to the extent that the Board found that the skilled artisan did *not* need to be skilled in synthetic nucleotide chemistry, this finding was unsupported by substantial

evidence, in light of the critical role that chemical synthesis plays in this case. A2174; A3801; *see Daiichi I*, 501 F.3d at 1256-1257; *DyStar*, 464 F.3d at 1361-1363. These errors led the Board to disregard Dr. Weinstock's inability to opine on matters of synthetic nucleotide chemistry, as reflected throughout its factual findings.

B. Labeling The Base Was Not Obvious

The Board's first inferential leap was that a skilled artisan would have been motivated to try a method with a reversible chain-terminating nucleotide with a label attached to the *base*, rather than to the cap on the *3'-OH group of the sugar*, and would have had a reasonable expectation of success in doing so. *See InTouch*, 751 F.3d at 1347. No substantial evidence supports those findings.

At the time of Dr. Ju's invention, SBS researchers considered nucleotides with labeled 3′-OH caps far more promising than base-labeled nucleotides. Tsien prefers to attach the label to the cap on the 3′-OH group. A3818-3819; A3841-3842. Tsien features such a nucleotide both in its "examples" and in its detailed description of nucleotide labeling. A3027:1-3028:32; A3036:1-3040:22; A3051-3058; *see* A3818-3820; A3442:7-16. Similarly, the sequencing apparatus embodied in Tsien's figure 2 uses nucleotides with labels on their 3′-OH caps.

A3014:14-19; *supra* p.21 (demonstrative).[7] Seela, Prober, and Rabani do not address base-labeled nucleotides with removable 3′-OH caps at all. *See* A3833; A3839; A3850. Dr. Trainor therefore concluded that a skilled artisan "would have understood the 3′-OH labeled nucleotide analogues of Tsien to be the starting point for any further work to design and synthesize new nucleotide analogues." A3819-3820.

The Board, however, ignored this marked preference in Tsien—a view born of "hindsight bias" and inconsistent with this Court's admonition that "[p]otent and promising activity in the prior art trumps mere structural relationships" when reconstructing a skilled artisan's choice of starting compounds. *Daiichi Sankyo Co. v. Matrix Labs., Ltd.*, 619 F.3d 1346, 1354 (Fed. Cir. 2010). This Court has observed that "'a person of ordinary skill in the art would not select [particular

[7] The figure indicates that a unique label is used for each base by placing a different number of apostrophes after the letter indicating the base in each nucleotide—for example, "3′-blocked dA′TP" and "3′-blocked dC′′TP." The specification clarifies, however, that the placement of the apostrophe does not necessarily reflect the physical position of the label. A3011:7-14. A skilled artisan would therefore have turned to the text describing the figure to see where the label is attached and would have learned that the label is attached to the 3′-OH cap. A3014:14-19; A3818; A3542:9-14; *see supra* p.21. The Board apparently misunderstood the figure as depicting labels attached to the bases. A12.

references] as leads only to disregard one of their distinguishing characteristics'"—in this case, the labeled 3′-OH cap. *Id.* at 1356.[8]

The Board, moreover, shifted the burden of persuasion to Columbia, faulting Columbia for (supposedly) producing insufficient evidence that a base-labeled embodiment *would not* have been chosen by a skilled artisan. A14 ("[N]o specific disclosure has been identified in Tsien by Columbia which disparages these alternative [base-labeled] nucleotide analogues, or which would have led one of ordinary skill in the art to conclude that they were unsuitable for the SBS purpose described by Tsien."); A24 (similar). The Board thus ignored Congress's clear directive that the petitioner bear "the burden of proving … unpatentability by a preponderance of the evidence." 35 U.S.C. § 316(e).

Furthermore, the Board never addressed whether a skilled artisan would have had a *reasonable expectation of success* in making a base-labeled, 3′-OH-capped nucleotide. *InTouch*, 751 F.3d at 1347; *Otsuka*, 678 F.3d at 1292. As

[8] Dr. Weinstock, certainly, did not explain why a skilled artisan would have ignored Tsien's preference for a labeled 3′-OH cap in favor of a labeled base. Tellingly, when Illumina argued in its reply that base-labeled nucleotides would be "a natural choice for further development efforts in view of Tsien's teachings about label positioning and enzymatic competence," it cited none of Dr. Weinstock's testimony. A2255. It relied, instead, on its "rebuttal" expert, Dr. Burgess, whose testimony the Board did not consider, and on a portion of Dr. Trainor's deposition in which Dr. Trainor merely acknowledged that Tsien prefers a *removable* label, whether attached to the 3′-OH cap or elsewhere on the nucleotide, over *a non-removable label*. A4309:19-4311:9.

noted, Tsien provides instructions only for synthesizing Tsien's preferred 3′-OH-labeled embodiments. A3036:1-3038:11; A3058; *see* A3819-3820. Dr. Trainor testified that this approach would have been "synthetically attractive" because it required modifying fewer parts of the nucleotide. A4668:14-4669:5. The Board did not address the artisan's expectation of success in making a base-labeled alternative. The Board noted that Tsien "describes nucleotides with a cleavable linker and 3′-OH removable blocking group" (A27), but this is a non-sequitur, saying nothing about base-labeling. The Board also stated, incorrectly, that the question of the artisan's reasonable expectation of success had been "addressed above." *Id.*[9]

Had the Board addressed this question, it could only have ruled in Columbia's favor. Illumina's witness, Dr. Weinstock, disavowed any expertise in synthetic nucleotide chemistry and was therefore unqualified to opine on this issue. *See supra* p.25.

[9] The Board had earlier discussed the artisan's expectation of success in *incorporating a base-labeled nucleotide, once made, into a DNA strand*, but not the artisan's expectation of success in *making* such a nucleotide in the first place. A24-26. The issue of success in incorporating a nucleotide into a DNA strand is addressed below (at 47-48).

C. Using A Cleavable Linker Was Not Obvious

Even if a skilled artisan might have made a nucleotide with a 3′-OH cap and a base-label, attaching the label via a cleavable linker (as required by claim 15) would not have been obvious.

The Board acknowledged that Tsien's discussion of cleavable linkers does not describe using such a linker to attach a "label … to the base of the nucleotide as required by claim 15." A15. Nonetheless, the Board found that "[a] person of ordinary skill in the art reading Tsien would have recognized that its teaching of a cleavable [linker] to release the label would have been useful when the label is attached to the base." *Id.*; *see* A20-21.

The Board cited no expert testimony proffered by Illumina to support its view of what a skilled artisan would have "recognized" in Tsien. A15.[10] It thus appeared to rely on its own putative expertise and dispensed with its congressionally mandated role as an "impartial adjudicator." *Brand*, 487 F.3d at 869. An IPR, like a trial, is an "adjudicative," rather than an "examinational" proceeding, H.R. Rep. No. 112-98, at 46-47, and as such, the Board was required

[10] The Board did cite testimony from Dr. Trainor, but Dr. Trainor's testimony *contradicts* this finding. A21. In the cited testimony, Dr. Trainor acknowledged that Tsien discusses "the possibility of the label being attached to the nucleotide analogue by means of a cleavable tether." *Id.* However, Dr. Trainor also made clear that a skilled artisan would *not* have understood Tsien as teaching a nucleotide with both a cleavable linker and a base-label, and that the linkers discussed and illustrated in Tsien for attaching a label to a base are not cleavable. A3793-3794; A3831-3832; *see supra* p.22.

to rely on qualified expert evidence—rather than on its intuition—in assessing what the skilled artisan would have recognized. *See InTouch*, 751 F.3d at 1353. The Board relied on no such evidence; accordingly, its finding is unsupported by substantial evidence.

D. Using A Deazapurine Was Not Obvious

The Board's third inferential leap was that a skilled artisan would have used a deazapurine in place of a natural purine base.

1. No motivation to combine

The Board wrongly concluded that a skilled artisan would have been motivated to try an SBS method using base-labeled, 3′-OH-capped nucleotides with a deazapurine base. The Board mistakenly concluded that Tsien "reasonably suggested" labeling the C-7 position of a deazapurine base by citing to Prober (A19), even though Tsien advocated labeling the C-8 position of a natural purine base and nowhere disclosed the C-7 deazapurine alternative. That finding is unsupported by substantial evidence, for several reasons.

First, the Board erred to the extent that it relied on Dr. Weinstock in this context because Dr. Weinstock was, by his own admission, unqualified to opine on matters of synthetic nucleotide chemistry. *See supra* p.25. Dr. Trainor, who was indisputably qualified to testify on these matters, was adamant that a skilled artisan

"would not have deviated from Tsien's guidance to use the 'ideal' [C-]8-position." A3831.

Second, Tsien does *not* cite Prober for labeling purines. As explained above (at 22), Tsien teaches, while discussing his non-preferred base-labeled embodiment, that it would be "ideal" to attach the label at the C-8 position of a natural (that is, non-deaza-substituted) purine, as taught by a different reference. A3030:3-4; *see* A3829-3831. Tsien then cites Prober only for labeling the *pyrimidines* "thymidine and deoxycitidine." A3030:10-14. Accordingly, the diagrams in Tsien of base-labeled purine nucleotides show the label attached to the C-8 position of a natural purine. A3031; *see* A3829; A3443:22-3444:5; A3446:9-15; A3453:23-25. Rather than presenting a "generic disclosure" of a base-labeled nucleotide (A19), Tsien selects a particular scheme for achieving such labeling—a scheme that does not use a deazapurine.

Third, the Board never identified any affirmative motivation that would have led a skilled artisan to abandon the "ideal," natural C-8 position taught by Tsien (A3030:3-4) and pursue an alternative labeling position—the C-7 position of a deazapurine. The Board relied on Dr. Weinstock's testimony that modifying Tsien to use a deazapurine amounted to "'the use of the known techniques of Prober … to improve similar Tsien systems and methods *in the same way that the known features improve the methods and reagents of Prober.*'" A13 (emphasis added).

But neither the Board nor Dr. Weinstock identified the supposed "improve[ment]" that the skilled artisan would have been seeking. *Id.*; *see* A3181-3182. Even if "one of ordinary skill in the art *could* combine these references," Illumina failed to establish that the skilled artisan "*would* have been motivated to do so." *InTouch*, 751 F.3d at 1352.

Fourth, even if a skilled artisan had identified some "improve[ment]" in Prober, the skilled artisan would not have been motivated to combine Tsien with Prober because the "'systems and methods'" of Tsien and Prober are not "'similar.'" A13. The labeled deaza-nucleotides disclosed by Prober are irreversible chain-terminating ddNTPs for Sanger sequencing, where the label must remain attached during the harsh conditions of electrophoresis; these labeled ddNTPs cannot be used in SBS. A3826; A3830-3831. Therefore, "a [skilled artisan] would not have looked to … the description of *irreversibly* terminating … *ddNTPs* in Prober … to design new *dNTP* analogues for use as *reversible* … chain terminators." A3826 (emphases added).

Fifth, the Board erred by shifting the burden to Columbia to prove that Tsien taught away from the use of a deazapurine. A19-20. Columbia was not required to make such a showing in the absence of any proof by Illumina that the skilled artisan would have been motivated to combine the teachings of the prior art references. *InTouch*, 751 F.3d at 1347.

The Board made no separate findings with respect to Tsien and Seela, concluding that "[t]he reason for combining the cited publications is the same as for Tsien and Prober." A28. Its rejection on Tsien/Seela should be reversed for the same reasons discussed above for Tsien/Prober. Indeed, there is even less reason to combine the teachings of Tsien and Seela: The Board relied on Tsien's citation to Prober in finding a motivation to combine those references (A13; A18-19), but Tsien does not cite Seela at all. *See* A3847-3848. Even setting that aside, Dr. Trainor explained that a skilled artisan would not have been motivated to combine Tsien with the deazapurine teachings of Seela because Seela teaches deaza-substituted nucleotides (without labels or 3′-OH caps) for use in *Sanger sequencing* to address problems associated with electrophoresis, which is not used in Dr. Ju's claimed method. *See* A3850; *supra* pp.19-20.

2. No reasonable expectation of success

The Board also wrongly concluded that a skilled artisan would have had a reasonable expectation of success in combining Tsien with Prober or with Seela to synthesize a 3′-OH-capped nucleotide with a label attached to a deazapurine base (via a cleavable linker, for claim 15).

Dr. Trainor, who co-authored the Prober reference (A3781), testified in detail that a skilled artisan would "have had no basis for reasonably expecting that any combination of the disclosures of Tsien and Prober … could have been used

successfully to make" the claimed nucleotides. A3826; *see* A3834-3837. Dr. Trainor explained that because the deaza-nucleotides of Prober are *irreversible* chain-terminating *ddNTPs*, a skilled artisan who set out to "combin[e] the descriptions in Tsien and … Prober" to achieve Dr. Ju's invention would "have to create completely new chemical procedures to obtain a nucleotide analogue having the combination of features described in the claims." A3835. Dr. Trainor testified that "[c]reating such new chemical procedures is complex and fraught with difficulties," that "[t]he result of each step in such a procedure cannot be predicted," and that "[r]eaction conditions suitable for making one nucleotide analogue may be inapplicable to making another analogue." A3836.[11]

The Board did not make any findings on this issue.[12] Had the Board made this legally mandated inquiry, however, it would have had no basis for

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[11] Illumina argued below the '698 patent takes the synthesis of labeled deazapurines as "well accepted." A2261. But the '698 patent teaches that labeled, reversible chain-terminating deaza-dGTPs may be made by a method based on a combination of Prober and several other references not included in the grounds of review instituted by the Board. A143-144 (24:64-25:17). It does not discuss the combination of Prober and Tsien. In any event, "'[c]are must be taken to avoid hindsight reconstruction by using the patent in suit as a guide through the maze of prior art references.'" *In re NTP, Inc.*, 654 F.3d 1279, 1299 (Fed. Cir. 2011); *see Eli Lilly & Co. v. Teva Pharm. USA, Inc.*, 619 F.3d 1329, 1340 (Fed. Cir. 2010); *Ortho-McNeil Pharm., Inc. v. Mylan Labs., Inc.*, 520 F.3d 1358, 1364 (Fed. Cir. 2008); *In re Ochiai*, 71 F.3d 1565, 1570 (Fed. Cir. 1995).

[12] The Board did not make any separate findings as to Tsien and Seela, and thus also did not address whether a skilled artisan would have reasonably expected to succeed in making the nucleotides of Dr. Ju's claimed method based on those

disregarding Dr. Trainor's expert opinion. When Dr. Weinstock was asked whether he was "aware of any description of any synthetic chemistry that would permit [a skilled artisan] to add the labels … of Prober to" a *dNTP*, rather than a *ddNTP*, he admitted that he lacked expertise to "comment on the chemistry." A3414:6-12. Indeed, Dr. Weinstock repeatedly disavowed any expertise on the subject of nucleotide synthesis. *See supra* p.25. Therefore, the only evidence in the record before the Board indicated that a skilled artisan would not have had a reasonable expectation of success in making the claimed nucleotide by "combining" Tsien and Prober.

E. A Skilled Artisan Would Not Have Reasonably Expected The Nucleotides Of The Claimed Method To Be Incorporated By A Polymerase

Incorporation by a polymerase into a primer extension strand is central to the invention of the '698 patent and is expressly required by all of the challenged method claims. A149 (35:11); A3799. Designing a nucleotide that can be recognized by a polymerase and incorporated into a DNA strand is one of the most critical and unpredictable challenges in the field of SBS. A3799-3800; A3837-3838; A3854-3855. Because of the unpredictability of nucleotide recognition and incorporation, a skilled artisan would have hewn closely to approaches that had

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references. A28. Dr. Trainor testified that a skilled artisan would have faced the same synthetic chemistry hurdles in combining Tsien and Seela as in combining Tsien and Prober. *See* A3845-3846; A3851-3852.

previously shown promise—in particular, the approach preferred by Tsien of placing the label on the 3′-OH cap, so as to minimize "disruption to the nucleotide structure." A3819; *see* A3819-3820; A3825; A3836-3838; A3845-3846; A3853-3855. Dr. Trainor testified that a skilled artisan would not have expected the nucleotide analogue of Dr. Ju's claimed method to be incorporated, dismissing such an expectation as "totally speculative and unsupported by any scientific reason or literature." A3836-3837; A3853.

The Board's contrary conclusion is unsupported by substantial evidence. A24-26. The Board stated that Prober "teaches that base labeled nucleotides can be incorporated," and that "Dr. Trainor admitted that 3[′]-OH removably capped nucleotides had been used in DNA sequencing methods." A25. But the nucleotides in Prober are *irreversible chain-terminating* ddNTPs, rather than *reversible dNTPs*, and Dr. Trainor's cited testimony was expressly limited to *non-base-labeled* nucleotides. A3792-3794. The Board cited no other evidence to support its conclusion that these two generalized and disconnected observations supported a reasonable expectation for successful incorporation of Dr. Ju's base-labeled, reversibly capped nucleotides. Instead, it shifted the burden to Columbia to demonstrate why "two structures which are known to work … would not work when combined in the same nucleotide." A25. It thus disregarded its obligation to evaluate obviousness from the perspective of a skilled artisan, and again relieved

Illumina of its burden of proving a reasonable expectation of success by a preponderance of the evidence. *See* 35 U.S.C. §§ 103, 316(e); *InTouch*, 751 F.3d at 1347; *Otsuka*, 678 F.3d at 1292. In any event, Dr. Trainor *did* explain why incorporation was unpredictable, and why any expectation of success would have been "totally speculative and unsupported." A3836-3837; A3853.

F. Using Dr. Ju's Nucleotides To Sequence A Plurality Of Different Nucleic Acid Templates In A Microarray Was Not Obvious

Finally, the Board erred in considering the obviousness of using Dr. Ju's claimed method to sequence "a plurality of different nucleic acid templates" in a "microarray."[13] The Board did not substantively discuss these claim features in its final decision. In its Tsien/Prober obviousness rejection, the Board considered claim 11 separately but did not substantively address the obviousness of sequencing a "plurality of [different] nucleic acid templates." A27.[14] When it

[13] Sequencing "a plurality of different nucleic acid templates" is a step of the method of claim 5. A149 (35:34-35). Claim 11 recites a product comprising "[a] plurality of nucleic acid templates." *Id.* (36:24). Dr. Trainor and Dr. Weinstock agreed that claim 11 would have been understood by a skilled artisan as the product of claim 5, and thus as also requiring a plurality of *different* nucleic acid templates. A3816-3817; A3332:22-3333:19. In denying Columbia's motion to amend claim 11, the Board appeared to agree that adding "different" would not change the scope of the claim. A43. Claim 12 specifies that the plurality of nucleic acids is "present in a microarray." A149 (36:33).

[14] The Board stated that "Columbia did not separately argue claim 11" (A27), but that is mistaken. Columbia did separately argue this claim, albeit under the rubric of amended claim 25—*i.e.*, the proposed amended version of the claim clarifying that *different* templates are present. A2191. Dr. Trainor separately

turned to Tsien, Prober, and Rabani, the Board stated that "Columbia did not … identify a defect in the factual findings or reasoning which led to the institution of [this] patentability challenge," and the Board "adopt[ed] the findings and reasoning" of its decision to institute without further discussion. A27-28.

That holding was doubly wrong. First, in adopting "findings" the Board made in its decision to institute review, the Board disregarded the different standard of proof between the decision to institute review ("reasonable likelihood") and the final decision ("preponderance of the evidence"). 35 U.S.C. §§ 314(a), 316(e); 37 C.F.R. § 42.108(c). The decision to institute is *not* a prima facie finding of unpatentability, and Columbia did not have the burden to "identify a defect" in that decision. Rather, the burden remained squarely on Illumina to demonstrate why using the claimed method to sequence a plurality of different nucleic acid templates in a microarray would have been obvious in view of the asserted references.

Second, no substantial evidence supported the "findings" the Board adopted from its decision to institute. For the teaching in the prior art of sequencing a plurality of different nucleic acid templates, Illumina relied in its petition on a passage in Tsien discussing "[a] plurality of copies of a … DNA" molecule

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opined on the nonobviousness of the claims reciting a plurality of different nucleic acid templates in a microarray. A3838-3839; A3855.

immobilized on a surface, depicted in Tsien's figure 2. A2027; *see* A3012:34-36; A3053. Dr. Weinstock, however, conceded that the cited passage in Tsien refers to copies of "a single" template, not a plurality of *different* templates. A3516:4-8. Dr. Trainor agreed and testified that it would not have been obvious, in view of Tsien and Prober (or in view of Tsien and Seela) to sequence a plurality of different templates. A3838-3839; A3855. Similarly, Dr. Trainor testified that the portion of Rabani that Illumina (and Dr. Weinstock) relied on as teaching a plurality of different templates in a "microarray" is actually a discussion of an array of "detection instrumentation," not an array of templates. *See* A2056; A3187; A3840-3841; *supra* p.23.

* * *

Only through this sequence of unsubstantiated inferences and legal errors could the Board conclude that the claims are obvious, including over two pairs of references, Tsien with Prober and Tsien with Seela, that had been in the prior art for nearly a decade by the time of Dr. Ju's invention. As this Court has stated, "[i]f these discoveries and advances were routine and relatively easy, the record would undoubtedly have shown that some ordinary artisan would have achieved this invention within months of [the references]. Instead this invention does not appear for [nearly] a decade." *Leo Pharm. Prods., Ltd. v. Rea*, 726 F.3d 1346, 1354 (Fed. Cir. 2013). The Board's decision should therefore be reversed.

IV. THE BOARD WRONGLY DISREGARDED COLUMBIA'S COMPELLING EVIDENCE OF SECONDARY CONSIDERATIONS OF NONOBVIOUSNESS

The nonobviousness of Dr. Ju's invention was also forcefully demonstrated by Columbia's evidence that Illumina copied the invention, attempted to license it, and achieved commercial success by coopting it, and that the invention demonstrated unexpected advantages. The Board's contrary conclusion cannot be reconciled with the record evidence. A28-39.

This Court "has emphasized that consideration of the objective indicia is *part of* the whole obviousness analysis, not just an afterthought." *Leo Pharm. Prods.*, 726 F.3d at 1357. "Secondary considerations evidence can establish that 'an invention appearing to have been obvious in light of the prior art was not' and may be 'the most probative and cogent evidence in the record.'" *Apple Inc. v. ITC*, 725 F.3d 1356, 1366 (Fed. Cir. 2013). Although "the patentee in the first instance bears the burden of coming forward with evidence" demonstrating the existence of a "nexus" between the secondary considerations and the patented invention, this burden on the patentee is merely a burden of production; once the patentee has put forward such evidence, the burden of persuasively rebutting this evidence lies upon the challenger. *Demaco Corp. v. F. Von Langsdorff Licensing Ltd.*, 851 F.2d 1387, 1392-1393 (Fed. Cir. 1988); *see In re Cyclobenzaprine Hydrochloride Extended-Release Capsule Patent Litig.*, 676 F.3d 1063, 1078 & n.5 (Fed. Cir. 2012); *Crocs, Inc. v. ITC*, 598 F.3d 1294, 1311 (Fed. Cir. 2010). The challenging party may

attempt to carry its burden by proving that the objective indicia of nonobviousness, such as commercial success, were due "*exclusively* … to a feature that was 'known in the prior art." *Rambus*, 731 F.3d at 1257 (emphasis added). But the fact-finder must not lose sight of the requirement that "the obviousness inquiry centers on whether 'the claimed invention *as a whole*' would have been obvious." *Id.* at 1257-1258 (emphasis added).

The evidence demonstrates compellingly that Illumina consistently and unabashedly recognized the merits of Dr. Ju's claimed invention. When Dr. Ju's application was first published, Illumina's predecessor-in-interest copied the claimed invention; Illumina then spent seven years trying, unsuccessfully, to license what Illumina itself described as Dr. Ju's "[redacted]" (A4031); and, having failed in its licensing efforts, Illumina nonetheless went to market with products that embodied the claimed invention, achieving considerable commercial success due to the claimed features. Also, Dr. Ju's invention was unexpectedly better than the closest prior art, pyrosequencing.

Faced with this evidence of nonobviousness, the Board should have affirmed the patentability of the challenged claims. The Board reached a contrary conclusion only by committing a series of legal and factual errors.

A. Illumina's Predecessor Copied Dr. Ju's Patents

"The copying of an invention may constitute evidence that the invention is not an obvious one. This would be particularly true where the copyist had itself attempted for a substantial length of time to design a similar device, and had failed." *Vandenberg v. Dairy Equip. Co.*, 740 F.2d 1560, 1567 (Fed. Cir. 1984) (citation omitted). Columbia's evidence demonstrates that Manteia studied Dr. Ju's patent applications to learn how to make nucleotides for SBS. [redacted].

As described above (at 12-13), two presentations produced by Manteia in 2003 show that Manteia turned to Dr. Ju's published patent applications to solve key problems in its SBS approach. In particular, Manteia relied on figure 1 of the '698 patent for the suggestion to attach a label to the base, rather than to the "very crowded" 3′ position, and Manteia cited his work for the suggestion to use a "small 3′-OH [blocking group]." A3970; A3892-3894. [redacted]

Based on this evidence, Columbia argued before the Board that [redacted]

[redacted]

[redacted]

[redacted]

[redacted]" A2203. The Board acknowledged Columbia's copying argument, but failed to address it in its written decision. A29.

The Board's legal error in failing to address Columbia's evidence of copying requires that its decision be set aside. "[W]hen secondary considerations are present, though they are not always dispositive, it is error not to consider them." *In re Kao*, 639 F.3d 1057, 1067 (Fed. Cir. 2011); *see Transocean Offshore Deepwater Drilling, Inc. v. Maersk Contractors USA, Inc.*, 617 F.3d 1296, 1305 (Fed. Cir. 2010); *Cyclobenzaprine*, 676 F.3d at 1075-1076. Illumina's reliance on Dr. Ju's invention refutes its claim that Dr. Ju's invention was obvious. *Cf. Apple*, 725 F.3d at 1366 n.4 (holding that "evidence of industry copying" can provide "strong evidence of nexus" in support of other secondary consideration). This Court should reverse the Board's decision.

B. Illumina's Efforts To License Dr. Ju's Invention Reflect "Respect For The Invention"

Illumina sought to license Dr. Ju's SBS technology multiple times over several years, in what can only be seen as a sign of "respect for the invention." *Apple*, 725 F.3d at 1375. These licensing efforts are particularly probative because

they were made when Illumina was "under no apparent threat of litigation." *Transocean*, 699 F.3d at 1353.

Columbia's unrebutted evidence, outlined above (at 13-15), showed that Illumina sought to license Dr. Ju's invention *before* it even had an SBS product to protect from litigation, when it still hoped that Dr. Ju's patents would supply the "key technological component[] that [it was] missing" and help Illumina develop its "'sequencing' approach." A3993; A3995. These initial licensing efforts spanned months and included personal outreach to both Dr. Ju and Columbia officials. A3997-4012. Columbia instead licensed Dr. Ju's technology exclusively to IBS. A4014. Illumina again attempted to license the invention from Columbia after this development (*id.*), while Illumina and IBS held direct discussions that featured IBS's "Columbia University license" (A4024). *See* A4016-4030.

Illumina did not deny that its licensing efforts reflected "respect for the invention," *Apple*, 725 F.3d at 1375, but rather tried, unsuccessfully, to exclude Columbia's evidence (A45). The Board wrongly discounted that evidence, on the grounds that "the invention recognized by Illumina as having merit [in its licensing attempts] is one which is described in Tsien," and that there was "insufficient evidence that Illumina's licensing strategy was driven by recognition of the merits of the claimed invention, rather than knowledge of a patent potentially covering their own product." A38. Both findings are legally and factually erroneous.

1. The Board erred in finding that Illumina sought to license "Tsien's" nucleotide

The Board's theory that "the invention recognized by Illumina as having merit is one which is described in Tsien with the removable 3[′-O]H capping group and base label" was legally flawed. A38. In the first place, Illumina itself never made such an argument (*see id.*), and the Board should not have made this argument on Illumina's behalf. *See* 35 U.S.C. §§ 312(a), 316(e); *Brand*, 487 F.3d at 869.

More broadly, the Board erred as a matter of law when it "too finely parsed" Illumina's licensing activities to identify which precise features of Dr. Ju's invention drew Illumina's interest. *Institut Pasteur v. Focarino*, 738 F.3d 1337, 1347 (Fed. Cir. 2013). Where "[t]he central success described in the patent is the one prior art hoped for and is captured in the claims at issue," the licensing activities "'provide probative and cogent evidence' of non-obviousness." *Id.* Although licensing evidence that "*exclusively* relates to a feature that was 'known in the prior art'" might be discounted for lack of a nexus to the invention, "the obviousness inquiry centers on whether 'the claimed invention *as a whole*' would have been obvious." *Rambus*, 731 F.3d at 1257-1258 (emphases added). The burden of demonstrating that Illumina's licensing efforts related exclusively to a feature that was known in the prior art lay with Illumina. *Cyclobenzaprine*, 676 F.3d at 1078 & n.5; *Crocs*, 598 F.3d at 1311; *Demaco*, 851 F.2d at 1392-1393.

After Illumina effectively conceded that it had no such evidence to present, the Board was legally bound to find that the invention sought by Illumina was the claimed invention as a whole.

The Board's theory that it was "Tsien's" nucleotide that Illumina "recognized … as having merit" is also unsupported. A38. First, Tsien did *not* disclose to the public a 3′-OH-capped, cleavably linked, base-labeled nucleotide. *See supra* pp.24-27. Second, Illumina was quite clear that what it wanted to license was "his [*i.e.*, Dr. Ju's] reversible terminator[]"—not a molecule disclosed by Tsien a decade earlier. A3995.

2. The Board erred by speculating that Illumina sought a license merely to avoid litigation

No evidence supports the Board's speculation that Illumina pursued a license to avoid the prospect of litigation, rather than because it recognized the merits of Dr. Ju's invention. A38. Illumina sought a license before it even entered the SBS market. It was precisely because Illumina did not "have a play" in SBS, but knew that Dr. Ju had "solved the reversible terminator cleavable dye label issue," that Illumina became interested in licensing Dr. Ju's invention. A3993. The Board did not address this chronology. Moreover, as it did throughout its decision, the Board wrongly shifted the burden of persuasion to Columbia, discounting the licensing evidence because Columbia had (supposedly) not introduced sufficient evidence "that Illumina's licensing strategy was driven by

recognition of the merits of the claimed invention." A38. Illumina bore the burden of proving that its licensing efforts reflected something other than "respect for [Dr. Ju's] invention." *Apple*, 725 F.3d at 1375. It did not even attempt to do so.

C. The Blockbuster Commercial Success Of Products Embodying The Claimed Invention Demonstrates Its Nonobviousness

Commercial success of a product embodying an invention is powerful evidence of nonobviousness when "the commercial success … results from the claimed invention" and is "due to the merits of the claimed invention beyond what was readily available in the prior art." *J.T. Eaton & Co. v. Atlantic Paste & Glue Co.*, 106 F.3d 1563, 1571 (Fed. Cir. 1997). "When a patentee can demonstrate commercial success, usually shown by significant sales in a relevant market, and that the successful product is the invention disclosed and claimed in the patent, it is presumed that the commercial success is due to the patented invention." *Id.*; *see also Ormco Corp. v. Align Tech., Inc.*, 463 F.3d 1299, 1311-1312 & n.14 (Fed. Cir. 2006).

Columbia proved that Illumina made significant sales in the relevant market and that Illumina's products embody the claims of the '698 patent. A3879; A3880-3885; *see supra* pp.15-16. Illumina did not substantively contest this evidence in its reply. Columbia therefore made a "presumptive showing of nexus

between the commercial success and the claimed invention," *J.T. Eaton*, 106 F.3d at 1572; *see Ormco*, 463 F.3d at 1312, which Illumina did not rebut.[15]

The Board apparently credited Columbia's evidence tying the products' success to the claimed features of the invention, including a removable 3′-OH cap, a base-label, and a cleavable linker (as in claim 15). A35-37. Nonetheless, the Board criticized Columbia for failing to identify "any *other* feature of the invention as a whole that should be considered when evaluating commercial success" and concluded that "[t]he features … responsible for the commercial success of Illumina's product are … described and 'readily available' in Tsien." A37 (emphasis added).[16]

However, these claimed features are *not* "'readily available' in Tsien" (A37), even if Tsien refers, in separate embodiments, to a base-label, a 3′-OH cap, and a cleavable linker. (Tsien nowhere refers to a deazapurine.) This Court's precedent makes clear that a combination of features is not "readily available in the prior art," *J.T. Eaton*, 106 F.3d at 1571, if the features are merely disclosed

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[15] Illumina made no attempt to rebut Columbia's evidence that Illumina's products embody the claimed invention or to demonstrate that Illumina's commercial success is due to some other factor. The Board found that "Illumina marketed its SBS products as having … the same features embodied in claim 15." A36.

[16] In fact, Columbia *did* identify other claimed features of Dr. Ju's invention that Illumina itself described as responsible for its commercial success, including fluorescent labels, and successful incorporation into the DNA strand. *See* A3887-3888; A149 (35:11-13, 35:29-30).

separately in different parts of the same reference. Generally, a combination of features is "readily available" if the combination was found in a prior *commercial embodiment*. *See, e.g.*, *Therasense, Inc. v. Becton, Dickinson & Co.*, 593 F.3d 1289, 1299 (2010), *reinstated in relevant part*, 649 F.3d 1276, 1296 (Fed. Cir. 2011) (en banc); *Dippin' Dots, Inc. v. Mosey*, 476 F.3d 1337, 1345 (Fed. Cir. 2007); *Ormco*, 463 F.3d at 1312-1313. More rarely, a combination of features may be "readily available," despite not being previously commercialized, if it could be found in *a single embodiment* of a prior art reference. *See Asyst Techs. Inc. v. Emtrak, Inc.*, 544 F.3d 1310, 1316 (Fed. Cir. 2008). This Court has never suggested that a combination may be "readily available in the prior art" merely because *each element was separately disclosed* in various embodiments of a prior art reference.

Here, Illumina's witness, Dr. Weinstock, admitted that the combination of a base-label and a 3′-OH cap was not commercially available before the '698 patent's priority date. A3559:18-3560:8. Indeed, even though nearly a decade of SBS research passed between Tsien's publication in 1991 and Dr. Ju's invention in 2000, at least one of the three major genome centers in the country was still using Sanger sequencing at the time of the invention. A3283:15-3284:5; *see* A3574:3-3577:2. Furthermore, the Board acknowledged that Tsien does not expressly "describe [a] label attached via a linker to the base of the nucleotide as required by

claim 15." A15. Therefore, the claimed combination of features that accounted for Illumina's commercial success was *not* "readily available" in Tsien.

In addition, the Board erred by "too finely pars[ing]" Columbia's evidence of commercial success to search for the single, isolated feature to which this evidence pertains, even though "[t]he central success described in the patent is the one prior art hoped for and is captured in the claims at issue." *Institut Pasteur*, 738 F.3d 1347. The search for a nexus, like every part of the obviousness analysis, "centers on whether 'the claimed invention as a whole' would have been obvious.'" *Rambus*, 731 F.3d at 1258 (quoting 35 U.S.C. § 103). As the entire body of evidence in this case demonstrates, it was the claimed features of Dr. Ju's invention "as a whole" that transformed base-labeled, 3′-OH-capped nucleotides from a theoretical notion to a blockbuster commercial success.

D. The Claimed Invention Was Unexpectedly Better Than The Closest Prior Art

Evidence of "some superior property or advantage that a person of ordinary skill in the relevant art would have found surprising or unexpected" demonstrates nonobviousness. *In re Soni*, 54 F.3d 746, 750 (Fed. Cir. 1995).

The Board acknowledged Columbia's evidence that the claimed method showed superior unexpected properties, and that those superior unexpected properties were "'made possible by the fact that Dr. Ju's nucleotide analogues separated the cleavable chemical group at the 3'-OH position of the sugar from the

detectable label, which was placed instead on the base.'" A30. This evidence was unrebutted by Illumina. The Board, however, suggested that the advantage was inherent in Tsien, even though "there is no working example in Tsien of a nucleotide with the claimed features." A31. Moreover, while the Board correctly noted that "[a] showing of 'new and unexpected results' must be 'relative to prior art'" (A33), it *sua sponte* determined that the closest prior art for comparison to Dr. Ju's claimed invention was not pyrosequencing—the only commercial embodiment of SBS at the time of Dr. Ju's invention (*see supra* p.12 & n.2), and the comparison that the '698 patent itself makes (A132 (2:18-32))—but rather the hypothesized and uncommercialized method practiced with nucleotides disclosed in Tsien, for which no comparable sequencing data was available. *See* A30. Illumina never advanced that argument, and no substantial evidence supports it.

CONCLUSION

The Court should reverse the Board's decision.

Respectfully submitted.

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September 29, 2014

CERTIFICATE OF SERVICE

I hereby certify that on this 29th day of September, 2014, I filed the foregoing Nonconfidential Brief for Appellant with the Clerk of the United States Court of Appeals for the Federal Circuit via the CM/ECF system and served a copy on counsel of record by the CM/ECF system.

/s/ Paul R.Q. Wolfson
PAUL R.Q. WOLFSON

CERTIFICATE OF COMPLIANCE

Pursuant to Federal Rule of Appellate Procedure 32(a)(7)(C), the undersigned hereby certifies that this brief complies with the type-volume limitation of Federal Rule of Appellate Procedure 32(a)(7)(B) and Circuit Rule 32(b).

1. Exclusive of the exempted portions of the brief, as provided in Federal Rule of Appellate Procedure 32(a)(7)(B), the brief contains 13,520 words.

2. The brief has been prepared in proportionally spaced typeface using Microsoft Word 2010 in 14 point Times New Roman font. As permitted by Federal Rule of Appellate Procedure 32(a)(7)(C), the undersigned has relied upon the word count feature of this word processing system in preparing this certificate.

/s/ Paul R.Q. Wolfson
PAUL R.Q. WOLFSON

September 29, 2014

ADDENDUM

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Paper 128
Mailed: March 6, 2014

UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

ILLUMINA, INC.
Petitioner

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner

Case IPR2012-00006
Patent 7,713,698 B2

Before SALLY G. LANE, RICHARD M. LEBOVITZ, and DEBORAH KATZ, *Administrative Patent Judges*.

LEBOVITZ, *Administrative Patent Judge*.

FINAL WRITTEN DECISION

35 U.S.C. § 318(a) and 37 C.F.R. § 42.73

I. BACKGROUND

A. Introduction

Petitioner, Illumina, Inc. ("Illumina"), filed a petition on September 16, 2012 ("Pet.), for *inter partes* review of claims 1-7, 11, 12, 14, 15, and 17 of U.S. Patent 7,713,698 B2 ("the '698 Patent") pursuant to 35 U.S.C. §§ 311-319 and 37 C.F.R. §§ 42.1 to 42.123. On March 12, 2013, the Board instituted *inter partes* review of claims 1-7, 11, 12, 14, 15, and 17 on three grounds of unpatentability (Paper 28, Decision on Petition ("Dec. Pet.")). Illumina requested rehearing on two of the grounds of unpatentability (Paper 30), which had been denied in the Decision on Petition. Upon reconsideration, the Board instituted *inter partes* review of one of these grounds of unpatentability as to claims 1-7, 11, 12, 14, 15, and 17 (Paper 43, Decision on Rehearing ("Dec. Reh'g")).

After institution of the *inter partes* review, Patent Owner, The Trustees of Columbia University in the City of New York ("Columbia"), filed a response under 37 C.F.R. § 42.120 to the decision instituting *inter partes* review (Paper 69, "PO Resp."). Columbia also filed a Motion to Amend Claims (Paper 70) and a Motion to Exclude Evidence (Paper 93). Illumina filed a reply to Columbia's response under 37 C.F.R. § 42.120 (Paper 76, "Pet'r Reply") and a Motion to Exclude Evidence (Paper 90 (redacted); Paper 107 (unredacted)). An oral hearing was held on December 17, 2013, with both parties in attendance. (Record of Oral Hearing, Paper 124.)

Among the evidence cited in this proceeding are declarations by George L. Trainor, Ph.D. (Ex. 2033, Trainor Decl.), on behalf of Columbia, and by George Weinstock, Ph.D. (Ex. 1021, Weinstock Decl.), on behalf of

Illumina. Dr. Trainor has a Ph.D. in Organic Chemistry and experience in DNA sequencing (Exhibit 2033, Trainor Decl. ¶¶ 3 and 6-8), qualifying him to testify on the issues discussed in his declaration. Dr. Weinstock has a Ph.D. in Microbiology and experience in DNA sequencing, including as a director of large-scale genome centers (Ex. 1021, Weinstock Decl. ¶¶ 4, 6, 8, and 9), qualifying him to testify on the issues discussed in his declaration.

The Board has jurisdiction under 35 U.S.C. § 6(c). This final written decision is issued pursuant to 35 U.S.C. § 318(a) and 37 C.F.R. § 42.73. Illumina has shown, by a preponderance of the evidence, that claims 1-7, 11, 12, 14, 15, and 17 are unpatentable.

B. The '698 Patent

The '698 Patent issued May 11, 2010. The named inventors are Jingyue Ju, Zengmin Li, John Robert Edwards, and Yasuhiro Itagaki. The invention of the '698 Patent involves sequencing DNA by incorporating a base-labeled nucleotide analogue into a primer DNA strand, and then determining the identity of the incorporated analogue by detecting the label attached to the base of the nucleotide. A polymerase is used to incorporate the nucleotide analogue into the strand of DNA ('698 Patent, col. 2, ll. 24-28). The method is generally referred to as "sequencing DNA by synthesis," or "SBS," because the sequence of the DNA is determined by identifying the successive additions of labeled nucleotides to a strand of DNA as it is synthesized, using a complimentary DNA strand as a template (*id.* at col. 2, ll. 6-11).

Columbia does not argue the novelty of the steps utilized in the claimed method of "determining the identity of a nucleotide analogue

incorporated into a nucleic acid primer extension strand… ('698 Patent, cl. 1)," but rather focuses its arguments on the novelty and non-obviousness of the nucleotide analogue utilized in the sequencing method. Nucleotides, which are the building blocks of DNA, comprise a sugar (ribose or deoxyribose), a phosphate attached to the 5'-position of the sugar, and a nitrogen base on the 1'-position of the sugar. During DNA synthesis, the 5'-position in the sugar of a new incoming nucleotide is linked by DNA polymerase to the 3'-OH group in the sugar of a preexisting nucleotide in the strand under synthesis. In order to identify the newly incorporated nucleotide, one approach described in the prior art is to attach a detectable label to the nucleotide at its 3'-OH group ('698 Patent, col. 2, ll. 33-37). For reference, the 3'-OH corresponds to 3'-position of the deoxyribose sugar of the nucleotide, and serves as the site where a new nucleotide is added during DNA synthesis.

The approach described in the '698 Patent is to make nucleotide analogues by linking a unique label, such as fluorescent dye, through a cleavable linker to the nucleotide base or to an analogue of the nucleotide base and to use a small removable chemical moiety to cap the 3'-OH group of the deoxyribose to make it reversibly nonreactive ('698 Patent, col. 2, ll. 57-65). The reason the 3'-OH group is made reversibly nonreactive is to allow the sequencing reaction to be terminated after each nucleotide is added in order to determine its identity (*id.* at col. 2, l. 64 to col. 3, l. 2). According to the '698 Patent, the prior art teaches attaching the label to the 3'-OH group. The '698 Patent, in contrast, puts the label on the nucleotide base and the removable chemical moiety on the 3'-OH group. These latter features are at the center of the patentability challenges.

All the claims at issue in this *inter partes* review involve a nucleotide analogue which comprises 1) a base labeled with a unique label, 2) a removable chemical moiety capping the 3'-OH group, and 3) a base which is deaza-substituted. A deaza-substituted nucleotide is a nucleotide analogue which includes a deazabase as the nitrogen base ('698 Patent, col. 7, ll. 44-63). A deazabase is a nitrogen base in which one of the natural nitrogen atoms in the base ring is substituted with a carbon atom (*id.*). For example, in a 7-deazapurine, the natural 7-position nitrogen in the base ring is replaced with a carbon atom (*id.*).

In summarizing the state of the art in Columbia's Patent Owner Response, Columbia states that, "[d]uring the 1990s, despite some interest in base-labeled nucleotide analogues, efforts focused on including a label on the 3'OH group on the sugar in a nucleotide analogue and on the design and synthesis of new nucleotide analogues that could be incorporated by a polymerase into a primer extension strand." (Paper 69, PO Resp. 8.) Columbia cites paragraphs 30-35 of Dr. Trainor's declaration as evidence that "[r]esults were mixed and it was recognized that new nucleotide analogues were needed [for use in] BASS [sequencing by synthesis; also known as SBS] sequencing." (*Id.*)

As discussed in more detail below, Columbia's characterization of the prior art as having "some interest in base-labeled nucleotide analogues" understates the interest level shown in the prior art. Tsien[1] and Dower,[2]

[1] Roger Tsien et al., WO 91/06678 (May 16, 1991), Exhibit 1002 ("Tsien").
[2] William Dower et al., U.S. Pat. No. 5,547,839 (August 20, 1996), Exhibit 1005 ("Dower").

cited in this *inter partes* review, and Stemple III,[3] cited in related proceedings, describe SBS methods, which disclose base-labeled nucleotides and nucleotides containing a removable chemical moiety at the 3'-OH position (Ex. 2033, Trainor Decl. ¶¶ 24 and 26-29). Columbia acknowledges that base-labeled nucleotides were described in the prior art (*id.* at 28). We understand it to be Columbia's position that because there is no single working example in the cited prior art of a nucleotide with the base-label and removable 3'-OH blocking group being used in a DNA sequencing reaction, the disclosure of such a nucleotide is somehow diminished and amounts only to "some interest." Columbia, however, has not identified where in the prior art a nucleotide with a label on the base and removable 3'-OH chemical moiety was so disparaged that a person of ordinary skill in the art would have been dissuaded from using it in SBS methods. To the contrary, the disclosure in several publications of nucleotides with a label on the nucleotide base with a removable 3'-OH group group (e.g., Tsien, Dower, and Stemple III) shows a recognition within the prior art that such nucleotides were useful and effective in SBS methods.

C. Related Proceedings

The '698 Patent is the subject of *The Trustees of Columbia University in the City of New York v. Illumina, Inc.*, 1:12-cv-00376-UNA, currently pending in the United States District Court for the District of Delaware

[3] Derek L. Stemple et al., U.S. Pat. No. 7,270,951 B1 (September 18, 2007), Exhibit 1008 ("Stemple III").

(Petition 3-4). According to Illumina, Columbia alleges in that proceeding that Illumina has infringed, and continues to infringe, the '698 Patent (*id*.).

There are two pending *inter partes* trials related to this trial:

A petition for *inter partes* review was filed on September 16, 2012, for U.S. Pat. No. 7,790,869 B2 ("the '869 Patent").[4] The '869 Patent is assigned to Columbia, has claims directed to related subject matter of the '698 patent, and has the same lineage as the '698 Patent. We instituted *inter partes* review on March 12, 2013.

A petition for *inter partes* review was filed on October 3, 2012, for U.S. Pat. No. 8,088,575 B2 ("the '575 Patent")[5] which is based on a continuation application of the '869 Patent. The '575 patent is assigned to Columbia and has claims directed to related subject matter of the '698 patent. We instituted *inter partes* review on March 12, 2013.

D. The Alleged Grounds of Unpatentability

We instituted *inter partes* review on the following four grounds of unpatentability:

I. Claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien and Prober I[6] (Petition 27).

II. Claims 5 and 12 under 35 U.S.C. § 103(a) as obvious in view of Tsien, Prober I, and Rabani[7] (Petition 52).

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[4] IPR2012-00007.
[5] IPR2013-00011.
[6] James M. Prober et al., *A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides*, 238 SCIENCE 336-341 (1987), Exhibit 1003 ("Prober I").

III. Claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien and Seela I[8] (Petition 56).

IV. Claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 102(b) as anticipated by Dower (Petition 30).

E. Claims

Claims 1 and 11 are the only independent claims under review. Claims 2-7, 14, 15, and 17 depend from claim 1. Claim 12 depends from claim 11.

Claims 1 and 11 are reproduced below:

> 1. A method of determining the identity of a nucleotide analogue incorporated into a nucleic acid primer extension strand, comprising:
>
> a) contacting a nucleic acid template attached to a solid surface with a nucleic acid primer which hybridizes to the template;
>
> b) simultaneously contacting the product of step a) with a polymerase and four nucleotide analogues which are either (i) aA, aC, aG, and aT, or (ii) aA, aC, aG, and aU, so as to incorporate one of the nucleotide analogues onto the nucleic acid primer and form a nucleic acid primer extension strand, wherein each nucleotide analogue within (i) or (ii) comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the nucleotide analogue, and wherein at least one of the four nucleotide analogues within (i) or (ii) is deaza-substituted; and
>
> c) detecting the unique label of the incorporated nucleotide analogue,

---

[7] Ely Rabani et al., WO 96/27025 (September 6, 1996), Exhibit 1006 ("Rabani").
[8] Frank Seela, U.S. Pat. No. 4,804,748 (February 14, 1989), Exhibit 1014 ("Seela I").

so as to thereby determine the identity of the nucleotide analogue incorporated into the nucleic acid primer extension strand.

11. A plurality of nucleic acid templates immobilized on a solid surface, wherein a nucleic acid primer is hybridized to such nucleic acid templates each such nucleic acid primer comprising a labeled incorporated nucleotide analogue, at least one of which is deaza-substituted, wherein each labeled nucleotide analogue comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the of the nucleotide analogue.

PATENTABILITY CHALLENGES

II. TSIEN AND PROBER I

We instituted *inter partes* review of claims 1-7, 11, 12, 14, 15, and 17 on the grounds that the claims would have been obvious under 35 U.S.C. § 103 in view of Tsien and Prober I. We first turn to the description in Tsien and Prober I of key elements of the claims, and then to the reason for combining Tsien and Prober I to have arrived at the claimed invention.

A. Claim 1 and others

Claim 1 is drawn to nucleic acid sequencing involving steps of: a) contacting a nucleic acid template with a primer; b) contacting the template hybridized with a polymerase and four nucleotide analogues, where each base has a unique label and a removable chemical moiety capping the 3'-OH group of the nucleotide sugar; and c) detecting the unique label of the nucleotide analogue which is incorporated into the primer as the primer is extended. At least one of the four nucleotide analogues is deaza-substituted.

A nucleotide analogue of claim 1 has the following structures or features: 1) a unique label attached to a base; 2) a removable chemical moiety capping the 3'-OH group of the nucleotide sugar; and 3) a deaza-substituted base.

Tsien

Tsien describes a DNA sequencing by synthesis method (Tsien, p. 6-7). The method uses nucleotides labeled with reporter groups to identify when they are incorporated into the newly synthesized strand (*id*. at p. 7, ll. 3-14).

The following evidence from Tsien supports Illumina's contention that features 1) and 2) are described in Tsien (*see also* Pet. 19-25).

1) Unique label attached to a base

Tsien has the following teachings:

> When they [deoxynucleotide triphosphates or dNTPs] are each tagged or labeled with different reporter groups, such as different fluorescent groups, they are represented as dA'TP, dC"TP, dG'"TP and dT""TP. As will be explained in more detail below, the fact that the indication of labeling appears associated with the "nucleoside base part" of these abbreviations does not imply that this is the sole place where labeling can occur. Labeling could occur as well in other parts of the molecule.

(Tsien, page 10, ll. 7-15 and Fig. 2.)

> While the above-described approaches to labeling focus on incorporating the label into the 3'-hydroxyl blocking group, there are a number of alternatives - particularly the formation of a 3'-blocked dNTP analogue containing a label such as a fluorescent group coupled to a remote position such as the base.

(*Id*. at 27, l. 33 to p. 28, l. 2.)

> One method involves the use of a fluorescent tag attached to the base moiety. . . . This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence.

(*Id.* at 28, ll. 5-6, 10-12.)

2) Removable 3'-OH chemical moiety (capping group)

During DNA synthesis, nucleotides are sequentially added to the 3'-OH group of the nucleotide sugar. The 3'-OH group contains a removable blocking group in Tsien's sequencing method so the labeled nucleotides can be added one at a time. After each addition, the label is detected and the 3'-OH group is deblocked and new nucleotide is added (Tsien, p. 13). Specifically, Tsien teaches:

> A deblocking solution is added via line 28 [Fig. 2] to remove the 3' hydroxyl labeled blocking group. This then generates an active 3' hydroxyl position on the first nucleotide present in the complementary chain and makes it available for coupling to the 5' position of the second nucleotide.

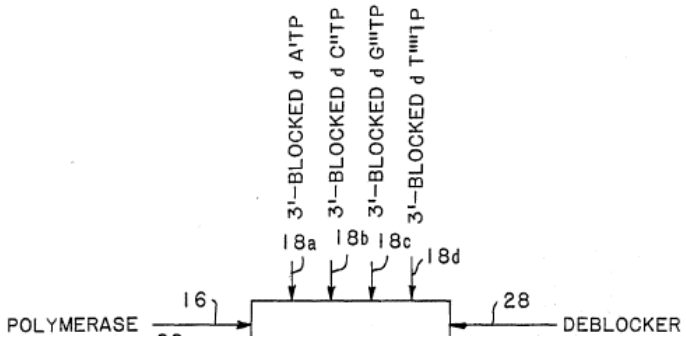
(Tsien, p. 13, ll. 17-22.)

> The coupling reaction generally employs 3' hydroxyl blocked dNTPs to prevent inadvertent extra additions [of nucleotides to the 3'-OH end].

(Tsien, p. 20, ll. 25-27.)

Structures 1) and 2) combined

Figure 2 of Tsien, reproduced below, shows nucleotides used in a sequencing reaction, each with a unique label and a blocked 3'-OH group (18a, 18b, 18c, and 18d) (Tsien, p. 12, ll. 14-18; p. 9, l. 35 to p. 10, 15):



A portion of Tsien's Figure 2,reproduced above, shows nucleotides each with a unique label attached to the nucleotide and a blocked 3'-OH group. The figure indicates that the labeling is on the base, but "these abbreviations [do] not imply that this is the sole place where labeling can occur." (Tsien, p. 10, ll. 7-15 and Fig. 2.)

3) A deaza-substituted base

Tsien does not disclose a deaza-substituted base, but references Prober I, which does. Specifically, Tsien teaches:

> One method involves the use of a fluorescent tag attached to the base moiety. . . . This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence. [citing Sarfati et al. (1987)] . . . . [Prober I] show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and Sequenase™.

(Tsien, p. 28, ll. 5-18.)

Prober I discloses the "set of four fluorescence-tagged chain-terminating reagents we have designed and synthesized is shown in Fig. 2A. These are ddNTP's to which succinylfluorescein has been attached via a linker to the heterocyclic base. . . . The linker is attached . . . to the 7 position in the 7-deazapurines. (Prober I, p. 337.) In sum, Prober I describes a

nucleotide comprising a deazapurine base to which a label has been attached.

Reason to combine

In making an obviousness determination, "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR Int'l Co. v. Teleflex, Inc.*, 550 U.S. 398, 418 (2007). Illumina contends that Tsien's reference to Prober I's fluorescent nucleotides would have provided one of ordinary skill in the art with a reason to have used Prober I's labeling technique in Tsien's method

> because the nucleotide analogues disclosed in Prober I, wherein "a linker is attached to the 5 position in the pyrimidines and to the 7 position in the 7-deazapurines," is shown to be an effective way to attach a fluorescent label to a nucleic acid base while maintaining the ability of the Sequenase™ polymerase used by Tsien to incorporate the associated dNTP into the primer extension strand.

(Petition 29.) Even absent disclosure of Prober I in Tsien, Dr. Weinstock testified that it would have been obvious to have used Prober I's teachings in Tsien.

> Prober I specifically teaches that nucleotide analogues incorporating 7-deazapurines may be used in sequencing reactions. Thus, the combination of Tsien and Prober I is the use of the known techniques of Prober I to improve similar Tsien systems and methods in the same way that the known features improve the methods and reagents of Prober I. Furthermore, use of the features taught by Prober I for their intended purpose, as disclosed by Prober I, would enhance the capability of the Tsien systems and methods in the same way they enhance the capability of the Prober I methods and reagents.

(Ex. 1021, Weinstock Decl. ¶ 66.)

Discussion

Columbia did not respond substantively to the patentability challenge of claim 1 based on Tsien and Prober I under 35 U.S.C. § 103 in either the Preliminary Response (Paper 21) or Patent Owner Response (Paper 69). However, in arguing for the patentability of a claim with narrower scope than claim 1 (i.e., proposed claim 18), Columbia contends that Tsien's base label nucleotide would not have been the "starting point" to make novel nucleotide analogues because of a preference for nucleotides with the label attached to the 3'-OH group (Paper 69, PO Resp. 18). We do not find this argument persuasive because there is an explicit description of base-labeled nucleotides in Tsien, and no specific disclosure has been identified in Tsien by Columbia which disparages these alternative nucleotide analogues, or which would have led one of ordinary skill in the art to conclude that they were unsuitable for the SBS purpose described by Tsien.

B. Claim 15

Claim 15 depends on claim 1 and further adds the limitation that "each of said unique labels is attached to the nucleotide analogue via a cleavable linker."

Although Illumina identified where in Tsien the "cleavable linker" limitation in claim 15 was described, Columbia did not separately address claim 15 in their Patent Owner Response. However, in the Motion to Amend the Claims, proposed new claim 18, which incorporates all the limitations of claim 15 into claim 1 (Paper 70, p. 4), was submitted by

Columbia along with arguments for its patentability over the cited art (Paper 69, PO Resp.). We consider these arguments below.

Tsien

Illumina cites the following passage in Tsien for a description of "a cleavable linker," as recited in claim 15 (Petition 26):

> In another type of remote labeling the fluorescent moiety or other innocuous label can be attached to the dNTP through a spacer or tether. The tether can be cleavable if desired to release the fluorophore or other label on demand. There are several cleavable tethers that permit removing the fluorescent group before the next successive nucleotide is added--for example, silyl ethers are suitable tethers which are cleavable by base or fluoride, allyl ethers are cleavable by Hg(II), or 2,4-dinitrophenylsulfenyls are cleavable by thiols or thiosulfate.

(Tsien, p. 28, ll. 19-29.)

Tsien, in this passage, thus describes a "space or tether" – the "linker" in claim 15 – which can attach the label to the nucleotide analogue ("dNTP" in Tsien). The tether is expressly taught by Tsien to "be cleavable if desired to release the fluorophore or other label on demand" and, therefore, is a "cleavable linker," as recited in the claim. This passage does not describe the label attached via a linker to the base of the nucleotide as required by claim 15, and claim 1 from which it depends. However, Illumina cited Tsien for its teaching "of a fluorescent tag attached to the base moiety" (Tsien, p. 28, ll. 5-6) to meet this limitation of the claim (Petition 21). A person of ordinary skill in the art reading Tsien would have recognized that its teaching of a cleavable tether to release the label would have been useful when the label is attached to the base moiety.

Columbia contends that the patentability challenge based on Tsien and Prober I is insufficient because "no starting point is identified and no rationale for the obviousness of the novel nucleotide analogue is provided." (Paper 69, PO Resp. 17.)

Columbia's argument is not persuasive. In the petition, Illumina cited Tsien's reference to Prober I for teaching labeled nucleotides and expressly stated that "Tsien thus provides an express teaching, suggestion, and motivation to combine Tsien with the disclosures of Prober I with respect to 'base moiety derivatized' nucleotide analogues." (Petition 28.) Furthermore, Illumina stated that Tsien teaches that "the synthesis scheme for ddNTPs used in Prober I should be used in Tsien to produce 'fluorescent dNTPs.' Tsien, p. 29, ll. 10-19." (*Id.*) Columbia's "starting point" argument is, therefore, unsubstantiated. A rationale to combine the publications was also described above in Section A based on testimony by Dr. Weinstock.

Columbia argues that if one of skill in the art would have used the base-labeled nucleotide analogues of Tsien as a "starting point," several differences between those nucleotide analogues and the claimed nucleotide analogues would "have had to be addressed." (Paper 69, PO Resp. 21.) Relying on Dr. Trainor's testimony, Columbia asserts that one of skill in the art would have had to make the following changes (Ex. 2033, Trainor Decl. ¶ 92):

> 1. remove the identical (non-unique) labels from the C-8 positions of the two purines despite the C-8 position being described by Tsien as the "ideal" position for the attachment of the labels to purines;
> 2. change the purine bases of the purines to deazapurines;
> 3. change the identical labels on the pyrimidines to unique labels;

> 4. replace the uncleavable, acetylenic linker (described in Prober I) on the pyrimidines with a cleavable linker;
> 5. replace the uncleavable alkylamino linker on the purines with a cleavable linker);
> 6. include removable 3'-OH capping groups on the uncapped 3'-OH groups of the nucleotide analogues; and
> 7. incorporate such a novel nucleotide analogue into the end of a primer extension strand.

(Ex. 2033, Trainor Decl. ¶ 92.)

We address each of these differences, below.

Deaza-substituted nucleotide (Nos. 1 and 2 in Ex. 2033, Trainor Decl. ¶ 92)

Citing the Trainor Declaration, Columbia argues "there was no reason to use a deaza-purine labeled at the 7-positiResn given Tsien's specific guidance to the contrary that a label on the 8-position of a non-deaza purine was 'ideal.' (Exhibit 2033, Trainor Decl., ¶§95-98 [])." (Paper 69, PO Resp. 22.) Columbia further argues that there would have been no reason "to change the uncleavable linkers on the 8-position of the purine labeled nucleotide analogues of Tsien to a cleavable linker, particularly since the linker in Prober I is uncleavable (Exhibit 2033, Trainor Decl., ¶98 [])" (*id.*).

Dr. Trainor cites Tsien's statement that the "C-8 position of the purine structure presents an ideal position for attachment of a label." (Tsien, p. 29, ll. 3-4.) Dr. Trainor acknowledges that Tsien cites Prober I in the same paragraph in which purine labeling is described and that Prober I describes producing labeled deazapurines (Ex. 2033, Trainor Decl. ¶ 96). However, Dr. Trainor states that Tsien ignored Prober I's teaching because Tsien "refers to Prober I for teaching an approach to producing fluorescently

labeled derivatives of pyrimidines." (*Id.*) The mentioned teaching in Prober I is reproduced below:

> A number of approaches are possible to produce fluorescent derivatives of thymidine and deoxycytidine. One quite versatile scheme is based on an approach used by Prober et al. (1987) to prepare ddNTPs with fluorescent tags.

(Tsien, p. 29, ll. 10-14.)

Columbia's argument is not persuasive or consistent with the full labeling disclosure in Tsien. Beginning at page 26, Tsien describes reporter groups on dNTPs and how they can be incorporated into a dNTP. Tsien states that one "approach employs fluorescent labels. These can be attached to the dNTP's via the 3'OH blocking groups or attached in other positions." (Tsien, p. 26, ll. 17-19.) After describing approaches to label the 3'-OH blocking group, Tsien goes on to state that "there are a number of alternatives - particularly the formation of a 3'-blocked dNTP analogue containing a label such as a fluorescent group coupled to a remote position such as the base. This dNTP can be incorporated and the fluorescence measured and removed according to the methods described below." (*Id*. at p. 27, l. 33 to p. 28, l. 4.) In the following paragraph, Tsien describes attaching a label to the base, and states:

> One method involves the use of a fluorescent tag attached to the base moiety. . . . This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence. Sarfati et al, (1987) demonstrates the incorporation of biotinylated dATP in nick translations, and other biotinylated derivatives such as 5-biotin (19)-dUTP (Calbiochem) are incorporated by polymerases and reverse transcriptase. Prober et al. (1987) [Prober I] show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and Sequenase™.

(*Id.* at p. 28, ll. 5-18.)

This passage, cited by Illumina on page 28 of the Petition, expressly mentions Prober I's method in its discussion of base labeling, reasonably suggesting that Tsien considered it suitable for Tsien's sequencing method. While Tsien discloses that the C-8 position of the nucleotide base is "ideal" for labeling a purine, that disclosure would not have dissuaded one of ordinary in the art from labeling at other positions in the base. "[J]ust because better alternatives exist in the prior art does not mean that an inferior combination is inapt for obviousness purposes." *In re Mouttet*, 686 F.3d 1322, 1334 (Fed. Cir. 2012). "A reference may be said to teach away when a person of ordinary skill, upon reading the reference, would be discouraged from following the path set out in the reference, or would be led in a direction divergent from the path that was taken by the applicant." *In re Gurley*, 27 F.3d 551, 553 (Fed. Cir. 1994). For a reference to "teach away" from using a particular approach, it must be shown that "the line of development flowing from the reference's disclosure is unlikely to be productive of the result sought by the applicant." *Id*. Dr. Trainor, himself, admitted that fluorescently labeled deazapurines had been used in the prior art (Ex. 2033, Trainor Decl. ¶¶ 20-21).

In this case, as mentioned above, there is generic disclosure in Tsien of labeling the base moiety, including a specific reference to Prober I, the latter describing C-7 deaza-labeled purine bases. Thus, even if labeling at the C-8 position is superior, Prober I's method is still reasonably suggested by Tsien, which characterizes Prober I as showing "enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and Sequenase™" (Tsien, p. 2, ll. 6-9; p. 19, ll. 9-18). Thus, those of skill in the art would have found the

use of Prober's analogues to be useful and effective, even if nucleotide analogues with a label on the 8-position of a non-deaza purine might have been better.

Unique labels (No. 3 in Ex. 2033, Trainor Decl. ¶ 92)

In his declaration, Dr. Trainor testifies that to have arrived at the claimed nucleotides from Tsien, a person of ordinary skill would have had to change the identical labels on the pyrimidines to unique labels. As explained by Illumina and in the section above on claim 1, Tsien has an express disclosure of using different reporter groups of each dNTP (*see* Section A). *See also* the following passage of Tsien:

> The detected florescence is then correlated to the fluorescence properties of the four different labels present on the four different deoxynucleotide triphosphates to identify exactly which one of the four materials was incorporated at the first position of the complementary chain. This identity is then noted.

(Tsien, p. 13, ll. 8-13.)

Thus, Tsien gives an express reason for using a unique label on each of the four different dNTPs: to identify what nucleotide is incorporated into the newly synthesized DNA molecule.

Cleavable linker between a base and a label in a nucleotide analogue (Nos. 4 and 5 in Ex. 2033, Trainor Decl. ¶ 92; original claim 15)

In claim 15 (and 18), the unique label is attached to the nucleotide by a "cleavable linker." As discussed above, Illumina argued that this limitation was described in Tsien, an assertion supported by the evidence. Columbia challenges that the limitation is met, arguing that "none of the

approaches to attaching labels to nondeazabases discussed by Tsien on page 29 at lines 3-18 and illustrated in the structures on page 30 involve use of a cleavable linker." (Ex. 2033, Trainor Decl. ¶ 98.)

Illumina did not argue that Tsien described cleavable linkers at the pages cited by Dr. Trainor, but rather cited page 28, lines 19-29, of Tsien for this disclosure as discussed above. Columbia contends there would have been no reason to change the uncleavable linkers on the 8-position of the purine labeled nucleotide analogues of Tsien to a cleavable linker, particularly since the linker in Prober I is uncleavable (Ex. 2033, Trainor Decl. ¶ 98, identified difference Nos. 4 and 5; Paper 69, PO Resp. 22). However, Tsien gives an express reason to use a cleavable linker when attaching a label to the deaza-substituted nucleotide: "to release the fluorophore or other label on demand." (Tsien, p. 28, ll. 22-23.) Dr. Trainor acknowledged in his declaration that Tsien describes "nucleotide analogues, which include a label attached to the base (Exhibit 1002, page 28, ll. 5-6) and the possibility of the label being attached to the nucleotide analogue by means of a cleavable tether (Exhibit 1002, page 28, ll. 19-21 [])." (Ex. 2033, Trainor Decl. ¶ 28 (emphasis added).) Accordingly, we are persuaded that Tsien teaches a cleavable linker.

"removable chemical moiety capping the 3'-OH group of the sugar" (No. 6 and 7 in Ex. 2033, Trainor Decl. ¶ 92)

In paragraph 92 of Dr. Trainor's declaration, he mentions one difference between Tsien and the claimed nucleotides as having to "include removable 3'-OH capping groups on the uncapped 3'-OH groups of the nucleotide analogues."

Dr. Trainor did not identify where uncapped 3'-OH groups were found in Tsien. The claims require a removable 3'-OH capping group. Tsien, as discussed above, also describes capped 3'-OH groups, a fact acknowledged by Dr. Trainor (Ex. 2033, Trainor Decl. ¶ 28). A blocking group on the 3'-OH is required to prevent inadvertent multiple additions (Tsien, p. 12, ll. 27-29).

The nucleotide analogues of Prober I are chain terminating and do not have an -OH group on the 3' carbon of the sugar (Prober I, Fig. 2). However, Tsien was relied upon for the 3'-OH capping group, not Prober I. Consequently, we find Dr. Trainor's testimony unavailing. Tsien teaches the nucleotides are added to the 3'-OH of the primer, extending it (Tsien, p. 11, 1-13; No. 6 and 7 in Ex. 2033, Trainor Decl. ¶ 92).

> *Was there a reason to move the label from the 3'-OH group to the base?*
>
> Columbia contends:
>
> > [T]here would have been no reason to change the preferred reversibly terminating 3'OH labeled nucleotide analogues of Tsien to move the label from the 3'OH group to the base since introducing modifications at two positions in a nucleotide analogue would have been understood by a person of ordinary skill to be more likely to result in a nucleotide analogue that a polymerase would not incorporate into a primer extension strand.

(Paper 69, PO Resp. 19.) Dr. Trainor testifies that having the label on the 3'-OH group "was to accomplish both labeling and removable capping at a single position on the nucleotide in a single series of chemical reactions." (Ex. 2033, Trainor Decl. ¶ 75.) Dr. Trainor states that there were no reports

of incorporating a nucleotide analogue into a primer, where the analogue had a removable cap on the 3'-OH group and a label on a base (*id.*)

As already discussed, Tsien expressly teaches placing the label on the base, rather than the 3'-OH group. Columbia's arguments to the contrary ignore the explicit disclosure by Tsien of base-labeled nucleotides. Moreover, Columbia's argument that a nucleotide with a label on the 3'-OH group is the appropriate starting point is factually incorrect because Tsien teaches nucleotides with the label on the base and the capping group on the 3'-OH. Even were there a preference for 3'-OH labeled nucleotides, this would not detract from the explicit disclosure of base-labeled nucleotides. Columbia's argument to the contrary is contradicted by the passages from Tsien reproduced below:

> As will be explained in more detail below, the fact that the indication of labeling appears associated with the "nucleoside base part" of these abbreviations does not imply that this is the sole place where labeling can occur. Labeling could occur as well in other parts of the molecule.

(Tsien, p. 10, ll. 10-15 (emphasis added).)

> One simple labeling approach is to incorporate a radioactive species within the blocking group or in some other location of the dNTP units.

(*Id.* at p. 26, ll. 13-15 (emphasis added).)

> Another labeling approach employs fluorescent labels. These can be attached to the dNTP's via the 3'OH-blocking groups or attached in other positions.

(*Id.* at p. 26, ll. 17-19 (emphasis added).)

> While the above-described approaches to labeling focus on incorporating the label into the 3'-hydroxyl blocking group, there are a number of alternatives - particularly the formation of

> a 3'-blocked dNTP analogue containing a label such as a fluorescent group coupled to a remote position such as the base.

(*Id.* at p. 27, l. 33 to p. 28, l. 2) (emphasis added).)

> One method involves the use of a fluorescent tag attached to the base moiety.

(*Id.* at p. 28, ll. 5-6 (emphasis added).)

Columbia attempts to distinguish Prober I because Prober I teaches chain terminating nucleotides which lack a removable group. But Prober I was only relied upon for its teaching of how to label a purine base with a detectable label. Tsien was relied upon for its teaching DNA sequencing using nucleotides with removable 3'-OH groups.

Dr. Trainor cited several publications for describing on-going efforts to create modified nucleotides with labels on the 3'-OH (Ex. 2033, Trainor ¶¶ 27, 28, and 86), said to teach against labeling the nucleotide base. Columbia's argument ignores explicit disclosure in Tsien of a base-labeled nucleotide. The fact that more than one type of nucleotide was being pursued for sequencing is not evidence that one approach would have been discouraged or abandoned over the other. We have not been directed to evidence that base-labeled nucleotides would have been ignored or seen as an unworkable alternative for use in sequencing by synthesis methods.

In addition to requiring a reason to have combined the prior art, the skilled worker must also have had a reasonable expectation of success of doing so. *Pfizer, Inc. v. Apotex, Inc.*, 480 F.3d 1348, 1361 (Fed. Cir. 2007). Columbia raises the issue of whether there would have been a reasonable expectation of success that a nucleotide analogue with a label on the base and a capping group on the 3'-OH would be incorporated into a DNA. A preponderance of the evidence supports an affirmative answer.

Prober I teaches that base labeled nucleotides can be incorporated into a newly synthesized DNA strand by appropriate enzymes (Prober I, p. 337, col. 2; p. 340, col. 1, second paragraph). Dr. Trainor admitted that 3'-OH removably capped nucleotides had been used in DNA sequencing methods (Ex. 2033, Trainor Decl. ¶¶ 26-28). Dr. Trainor cites several publications in support of unpredictability, but did not sufficiently explain the pertinence of these publications.

Dr. Trainor cites page 4263 of Metzker[9] (Ex. 2033, Trainor Decl. ¶ 112). On page 4263, Metzker describes testing 3'-OH modified terminators for their ability to be substrates for polymerases. As shown in Table 2 of Metzker, terminators had different activities when tested against various polymerases. Dr. Trainor did not explain how these results with different nucleotides than those which are claimed make it unpredictable that two structures which are known to work with polymerase would not work when combined in the same nucleotide molecule. In fact, the publication shows the routineness of testing for the ability of an analogue to be incorporated into DNA by a polymerase.

Dr. Trainor also cites page 3 of Canard and Sarfati (1994),[10] but without explaining its significance. The abstract of the paper describes synthesizing nucleotide analogs which "acted as substrates with several DNA polymerases leading to chain termination." Page 3 appears to describe some differences in the effectiveness of the synthesized nucleotides with the

[9] Michael L. Metzker et al., *Termination of DNA synthesis by novel 3'-modified-deoxyribonucleoside 5'-triphoshpates*, 22 Nucleic Acids Res. 4259-4267 (1994), Exhibit 2015.
[10] Bruno Canard et al., *DNA polymerase fluorescent substrates with reversible 3'-tags*, 148 Gene 1-6 (1994), Exhibit 2030.

different polymerases, but Dr. Trainor did not point to any specific instance or what relevance it had to unpredictability in view of the success pointed out in the abstract.

Finally, Dr. Trainor contrasts these publications with page 200 of Welch and Burgess (1999).[11] According to Dr. Trainor, Welsh showed that preliminary tests of compounds 1a and 1b as polymerase substrates did not show evidence of incorporation (Ex. 2033, Trainor Decl. ¶ 31). However, Dr. Trainor did not explain the pertinence of these compounds and their underlying chemistry to a nucleotide having a labeled deaza-purine and a removable 3'-OH group.

In sum, the preponderance of the evidence establishes that there was a reasonable expectation of success and Columbia has not directed us to sufficient evidence to establish that it was unpredictable to have used the claimed nucleotide as a polymerase substrate for DNA sequencing.

Was there a basis for reasonably expecting that a nucleotide with a removable 3'-OH group and a label attached to the base could be made?

Columbia contends that neither Tsien nor Prober I discloses any chemistry relevant to making a nucleotide analogue with the claimed features, requiring a person of ordinary skill "to design new chemical procedures to attempt to address the differences between the nucleotide analogues described by Tsien and the nucleotide analogue recited in the claim." (Paper 69, PO Resp. 20-21.) Furthermore, Dr. Trainor testifies that

[11] Mike B. Welch et al., *Synthesis of Fluorescent, Photolabile 3'-O-Protected Nucleoside Triphosphates for the Base Addition Sequencing Scheme*, 18 Nucleosides & Nucleotides 197-201 (1999), Exhibit 2027.

Prober I's nucleotides do not include a cleavable linker and cannot be modified to include, a 3'-OH group (Ex. 2033, Trainor Decl. ¶¶ 104-105). Dr. Trainor concludes that new chemical procedures would have been needed, the development of which were complex and fraught with difficulties (*id.* ¶¶ 106-107).

This argument is not persuasive. First, the patentability challenge is not based on converting Prober I's nucleotide into the claimed nucleotide. Rather, the analysis begins with Tsien who describes nucleotides with a cleavable linker and 3'-OH removable blocking group. Secondly, a preponderance of evidence establishes a reasonable expectation of success as addressed above.

C. Claim 11

Independent claim 11 is drawn to a plurality of nucleic acid templates hybridized with a primer, where the primer has incorporated a nucleotide which is 1) deaza-substituted; 2) has a based labeled with a unique label; and 3) has a removable chemical moiety capping the 3'-OH group of the sugar. All three structures present in the nucleotide have been discussed above and are described or suggested by the combination of Tsien and Prober I. Columbia did not separately argue claim 11 in their response under 37 C.F.R. § 42.120.

III. TSIEN, PROBER I, AND RABANI

We instituted *inter partes* review of claims 5 and 12 on the grounds that the claims would have been obvious under 35 U.S.C. § 103(a) in view of Tsien, Prober I, and Rabani (Dec. Pet. 29-30). Columbia did not in their

response under § 42.120 (PO Resp., Paper 69) identify a defect in the factual findings or reasoning which led to the institution of the patentability challenge. We therefore adopt the findings and reasoning set forth in the Decision on Petition.

IV. TSIEN AND SEELA I

We instituted *inter partes* review of claims 1-7, 11, 12, 14, 15, and 17 on the grounds that the claims would have been obvious under 35 U.S.C. § 103(a) in view of Tsien and Seela I (Dec. Pet. 26-28). Tsien has been discussed in detail above. In this challenge, instead of Prober I, Seela I was cited for its disclosure of deaza-modified nucleotides. The reason for combining the cited publications is the same as for Tsien and Prober (*id.* at 27-28).

Columbia relies upon the same arguments as made for Tsien and Prober I (Paper 69, PO Resp. 25). We find those arguments unpersuasive for the same reasons as above.

V. SECONDARY CONSIDERATIONS

The question of obviousness is resolved on the basis of underlying factual determinations including: (1) the scope and content of the prior art; (2) the level of ordinary skill in the art; (3) the differences between the claimed invention and the prior art; and (4) secondary considerations of nonobviousness, if any. *Graham v. John Deere Co. of Kansas*, 383 U.S. 1, 17 (1966). Secondary considerations are "not just a cumulative or confirmatory part of the obviousness calculus but constitute independent evidence of nonobviousness . . . [and] enable[] the court to avert the trap of

hindsight." *Leo Pharm. Prods., Ltd. v. Rea*, 726 F.3d 1346, 1358 (Fed. Cir. 2013) (internal quotation marks and citations omitted). "[E]vidence of secondary considerations may often be the most probative and cogent evidence in the record. It may often establish that an invention appearing to have been obvious in light of the prior art was not." *Stratoflex, Inc. v. Aeroquip Corp.*, 713 F.2d 1530, 1538 (Fed. Cir. 1983). "This objective evidence must be 'considered as part of all the evidence, not just when the decisionmaker remains in doubt after reviewing the art.' *Id.* at 1538-39." *Transocean Offshore Deepwater Drilling, Inc. v. Maersk Drilling USA, Inc.*, 699 F.3d 1340, 1349 (Fed. Cir. 2012).

Columbia contends that Illumina's obviousness challenges fail, because objective evidence shows: 1) the claimed invention has yielded unexpectedly improved properties and results not present in the prior art; (2) the claimed invention has received praise and awards; (3) the claimed invention is responsible for Illumina's commercial success; (4) Illumina copied the claimed nucleotide analogues; (5) others in the art were skeptical that the claimed nucleotides and methods would be successful; and (6) Illumina attempted to license the claimed nucleotides and methods (Paper 69, PO Resp., p. 26). We have considered this evidence along with all the other evidence before us, but do not find it persuasive.

A. "unexpectedly improved properties"

Relying on data in Ju's 2006 publication[12] in which sequencing of a 20 nucleotide template was accomplished using "four nucleotide analogues,

[12] Jingyue Ju et al., *Four-color DNA sequencing by synthesis using cleavable fluorescent nucleotide reversible terminators*, 103 Proc. Nat'l Acad. Sci.

each having both a unique detectable label attached through a chemically cleavable linker to the base (two pyrimidines and two deazapurines), and a chemically cleavable chemical group capping the 3'-OH group of the sugar," Dr. Trainor testified that that the properties of the claimed nucleotides "have revolutionized the DNA sequencing industry." (Ex. 2033, Trainor Decl. ¶¶ 203-204.) Specifically, Dr. Trainor testified that Ju's results show that the sequencing with the claimed nucleotides are unexpectedly better than pyrosequencing by facilitating clear identification of all 20 nucleotides in the DNA template while pyrosequencing did not (*id.* ¶¶ 205-206). Dr. Trainor testified that this "accurate identification was made possible by the fact that Dr. Ju's nucleotide analogues separated the cleavable chemical group at the 3′-OH position of the sugar from the detectable label, which was placed instead on the base" (*id.* ¶ 207). Dr. Trainor further cited additional publications said to have reported similar successes (*id.* ¶¶ 210-211).

Ju 2006 reported DNA sequencing in which "four nucleotides (A, C, G, and T) are modified as reversible terminators by attaching a cleavable fluorophore to the base and capping the 3-OH group with a small chemically reversible moiety so that they are still recognized by DNA polymerase as substrates." (Ju, p. 19635.) Dr. Trainor attributes Ju's success to this configuration, i.e., the label on the base and the 3-OH removable cap, but not to the deaza substitution. (Ex. 2033, Trainor Decl. ¶ 207). Claim 1 of the '698 Patent is drawn to a method which has a nucleotide with the removable capping group and the fluorophore label on the base, but does not require that the label be cleavable. Thus, the evidence is not commensurate

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19635-19640 (2006), Exhibit 2034. The Ju publication is said to correspond to the claimed invention with respect to the nucleotides and methods.

with the full scope of claim 1. Claim 15, on the other hand, requires that the label is attached to the nucleotide analogue via a cleavable linker – as in the nucleotide utilized in Ju's sequencing.

While there is no working example in Tsien of a nucleotide with the claimed features, as explained above, Tsien suggests attaching a label to the base moiety and utilizing a cleavable tether to release the label before the next successive nucleotide is added (Tsien, p. 28, ll. 5-25). Tsien's method also requires removable 3'-OH groups in its sequencing (*id*. at p. 21, ll. 9-12; p. 23, ll. 28-32). In considering the weight of the evidence militating in favor of the "unexpectedly improved properties" over pyrosequencing, we must take into account that a single reference describes both features, i.e., attachment of a label to the base and a cleavable linker as the attachment means. This implicates the legal principles enunciated in *In re Baxter Travenol Labs.*, 952 F.2d 388, 392 (Fed. Cir. 1991).

In *Baxter*, the applicant had argued that the claimed plasticized blood donor bag comprised of DEHP had unexpected properties in suppressing hemolysis of red blood cells stored inside it. *Baxter*, 952 F.2d at 389. The court found that such evidence did not rebut prima facie obviousness because the prior art disclosed a DEHP-plasticized donor bag, and therefore, Baxter's blood bag had the same hemolytic-suppressing function as the prior art – albeit unappreciated at the time of the invention. *Baxter*, 952 F.2d at 391. The court concluded that "[m]ere recognition of latent properties in the prior art does not render nonobvious an otherwise known invention." *Baxter*, 952 F.2d at 392. Likewise, Tsien has a written description of a nucleotide analogue with the features relied upon by Columbia as possessing unexpected properties. Thus, it could be said that the finding of a nucleotide

analogue with the 3'-OH and label on the base is better than pyrosequencing is merely recognizing an advantage of a nucleotide analogue described by Tsien. The description is not anticipatory to the Columbia claim because the claim further requires a deazapurine base. However, the deazapurine is not said by Columbia to be responsible for the unexpected result.

Dr. Trainor also testified that an "unexpected benefit" associated with the claimed nucleotide analogues was identified by Illumina's expert Dr. Weinstock (Ex. 2033, Trainor Decl. ¶ 213). According to Dr. Trainor, Dr. Weinstock stated during his deposition that "nucleotide analogues having a label on the base have the beneficial property of being useable in sequencing methods that require repetitive incorporation of nucleotide analogues, in particularly dGTPs, to sequence DNA having G:C rich regions." (*Id.*) Dr. Trainor stated that he "was surprised to learn that nucleotide analogues having a label on the base have solved the problem of sequencing G:C rich regions." (*Id.* ¶ 214.) This testimony is not persuasive.

Dr. Weinstock, in his deposition, specifically stated that Prober I had used "2'-deoxy-7-deazaguanosine triphosphates . . . in place of dGT to minimize" the effects of secondary structure when sequencing GC-rich regions. (Ex. 1034, Weinstock Dep. 141:5-18; 145:10-22.) Dr. Weinstock also testified that GC-rich regions "had a tendency to form secondary structures that were difficult for a DNA polymerase to get through during a DNA synthesis reaction and that the addition of deazabases to the end of the primer may have some benefit" in sequencing (*id.* at 147:8-13; *see also* 148:24 to 150:5). Based on this deposition testimony, it is evident that Dr. Weinstock believed that the problem of sequencing in GC-rich areas had already been addressed by Prober I in their use of the deazaguanosine,

inconsistent with Dr. Trainor's testimony that the problem was solved using analogues with a label on the base. Indeed, Dr. Weinstock's testimony is supported by Prober I, which taught that 2'-deoxy-7-guanosine triphosphates had been used to minimize secondary structure in sequencing (Prober I, p. 341, 1st column).

In response to questioning about the effect of a labeled deazabase, Dr. Weinstock added that "if a small change of substituting a carbon for a nitrogen has a benefit on reducing secondary structure in GC-rich regions, sticking anything larger than that at that position is likely to have an even bigger benefit." (Ex. 1034, Weinstock Dep. 151:13-21.) We understand Dr. Weinstock to be saying that further attaching a label to the deazapurine base would have been expected ("is likely") to have "an even bigger benefit" than the deazapurine alone which is inconsistent with Dr. Trainor's statement of unexpected benefit of the deazapurine labeled base. In sum, Dr. Weinstock's testimony is both credible and factually-supported.

A showing of "new and unexpected results" must be "relative to prior art." *Iron Grip Barbell Co., Inc. v. USA Sports, Inc.*, 392 F.3d 1317, 1322 (Fed. Cir. 2004). To establish unexpected results, the claimed subject matter must be compared with the closest prior art. *Baxter*, 952 F.2d at 392. In this case, Patent Owner's comparison was performed with pyrosequencing, but pyrosequencing is not the closest prior art. Rather, closer prior art is described in Tsien of a nucleotide with a label and removable group on the 3'-OH group. Patent Owner thus did not perform a comparison with the closest prior art.

B. Commercial success

Illumina sells products used in sequencing by synthesis (SBS), the same type of sequencing described in Tsien. Columbia introduced evidence that Illumina's SBS products included nucleotide analogues with a removable chemical moiety capping the 3'-OH group and a unique label on the base and that these features were "crucial to the commercial success" of Illumina's SBS products (Ex. 2033, Trainor Decl. ¶¶ 225-226). A nucleotide analogue with the latter two features is embodied by claim 15 of the '698 Patent. These Illumina products are also the subject of a patent infringement action by Columbia against Illumina (Paper 69, PO Resp. 40). In response, Illumina contends that Columbia has not presented any evidence supporting its commercial success argument (Paper 76, Pet'r Reply 14).

Commercial success involves establishing success in the marketplace of a product encompassed by the claims and a nexus between the commercial product and the claimed invention. "Evidence of commercial success, or other secondary considerations, is only significant if there is a nexus between the claimed invention and the commercial success." *Ormco Corp. v. Align Technology Inc.*, 463 F.3d 1299, 1311-12 (Fed. Cir. 2006). "For objective evidence to be accorded substantial weight, its proponent must establish a nexus between the evidence and the merits of the claimed invention." *In re GPAC Inc.*, 57 F.3d 1573, 1580 (Fed. Cir. 1995).

> While objective evidence of nonobviousness lacks a nexus if it exclusively relates to a feature that was "known in the prior art," *Ormco Corp. v. Align Tech., Inc.*, 463 F.3d 1299, 1312 (Fed. Cir. 2006), the obviousness inquiry centers on whether "the claimed invention as a whole" would have been obvious, 35 U.S.C. § 103.

Rambus Inc. v. Rea, 731 F.3d. 1248, 1257-58 (Fed. Cir. 2013).

With regard to whether a nexus has been established between the products upon which commercial success has been based and the claimed invention, Dr. Trainor testified that he reviewed Illumina's technical documents and that each of the nucleotide analogues "has a cleavably-linked label on the nucleotide base, namely a fluorescent dye molecule." (Ex. 2033, Trainor Decl. ¶ 231; *see also* ¶¶ 232-234.) Dr. Trainor also testified these commercial nucleotide analogs have a removable chemical moiety capping the 3′-OH group of the nucleotide sugar (*id.* ¶¶ 235-238). With regard to the deazapurine, Dr. Trainor reproduced a nucleotide which appears to be a C-substituted guanine at position 7 as it would be for a deazapurine, although Dr. Trainor did not provide specific testimony in support (*id*. ¶ 237). To the extent the nucleotides used by Illumina are not deazapurines, a nexus is not established because claims 1 and 15 require a deazapurine base.

As evidence that these features are responsible for the success of the commercial products, Dr. Trainor cited a February 17, 2006, email from Dr. Colin Barnes – a scientist at the predecessor company to Illumina – written to two other scientists at the same company. In the email, Dr. Barnes stated: "Our original concept of having a very small 3'-block and leaving the fluor on the base is the reason our SBS works so well." (Ex. 2033, Trainor Decl. ¶ 243 (emphasis omitted).) Dr. Barnes's email was written in 2006 at the time Mr. Sims[13] stated Illumina entered the SBS sequencing market with its

[13] Exhibit 2091 is the declaration of Raymond Sims which was provided by Columbia to establish commercial success of Illumina's products said to embody the claimed subject matter. Based on Mr. Sims's education and

nucleotide analogues having removable 3'OH groups and cleavable labels on the nucleotide base (Ex. 2091, Sims Decl. ¶ 14). Dr. Trainor also cited a deposition from Dr. Xiaohai Liu, Illumina's Director of SBS Sequencing Chemistry Research, who testified that he agrees with Dr. Barnes assessment. (Ex. 2033, Trainor Decl. ¶ 244; Ex. 2049, Liu Tr. 202:17-21.)

However, as held in *J.T. Eaton & Co., Inc. v. Atlantic Paste & Glue Co.*, 106 F.3d 1563, 1571 (Fed. Cir. 1997), "the asserted commercial success of the product must be due to the merits of the claimed invention beyond what was readily available in the prior art."

In this case, Dr. Trainor testified that "a nucleotide analogue combining all the features arranged as in Columbia patent claims – [(1)] a cleavable chemical group capping the 3′-OH position of the sugar and [(2)] a label attached to the nucleotide base via cleavable linker" were responsible for the nucleotides success. (Ex. 2033, Trainor Decl. ¶ 202; *see also* ¶¶ 226, and 229.) Dr. Barnes also attributed the success to these features. Illumina marketed its SBS products as having the cleavable label and removable 3'-OH group ("using a proprietary reversible terminator-based method that enables detection of single bases as they are incorporated into growing DNA strands. A fluorescently-labeled terminator is imaged as each dNTP is added and then cleaved to allow incorporation of the next base"), the same features embodied in claim 15 (*id*. ¶ 247). Both these features, however, are described in Tsien, making them known and "readily available in the prior art." The record indicates, therefore, that the success did not stem from the merits of the claimed invention. Neither Columbia, in their response under

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experience, we find him qualified to give opinions on financial data, the topic of his declaration.

§ 42.120 (Paper 69, PO Resp.), nor Trainor, in his declaration, described any other feature of the invention as a whole that should be considered when evaluating commercial success. *Rambus*, 731 F.3d at 1257-1258.

As discussed above, Tsien's nucleotides have a cleavable chemical group capping the 3′-OH position of the sugar in order to prevent inadvertent additions during the sequencing by synthesis method. A detectable label is described by Tsien on either the 3'-OH position or on the nucleotide base, and thus a nucleotide with label on the nucleotide base is one of two choices. The label on the nucleotide base is cleavable in order to identify subsequent nucleotide additions during the sequencing by synthesis method (Tsien, p. 13, ll. 1-29; p. 14, ll. 19-26; p. 17, ll. 14-16). The features said by Dr. Barnes, Dr. Liu, and Illumina to have been responsible for the commercial success of Illumina's product are thus described and "readily available" in Tsien. Indeed, Tsien's Figure 2 shows four unique labeled nucleotides, each with a removable 3'-OH blocking group and removable label (*id.* at p. 11, l. 28 to p. 13, l. 29). The removable label is depicted on the nucleotide base ("As will be explained in more detail below, the fact that the indication of labeling appears associated with the 'nucleoside base part' of these abbreviations does not imply that this is the sole place where labeling can occur." (*Id.* at p. 10, ll. 10-14.)).

C. Evidence of attempted licensing

Licensing of a patented technology can be evidence of non-obviousness because it can indicate the licensor recognizes the merits of the invention by licensing it. *Stratoflex*, 713 F.2d at 1539.

In this case, Columbia provided evidence that Illumina sought to license the technology developed by Dr. Ju (Paper 69, PO Resp. 37-39). Columbia states that it elected to license the technology to another company, not Illumina (*id.* at 39). Subsequently, Columbia states that Illumina had discussions about acquiring the company which gained a license to Ju's technology (*id.* at pp. 37-39). Columbia states that Illumina tried to acquire the licensed technology just prior to Columbia suing Illumina for patent infringement (*id.* at 40). Illumina did not challenge Columbia's description of its attempt to license the technology in their response to Columbia's § 42.120 filing. The only response was in their motion to exclude the evidence of attempted licensing as either hearsay or on lack of relevance.

Columbia has direct knowledge of Illumina's licensing attempts (Paper 69, PO Resp. 37-40). While Illumina never licensed the technology, Columbia argued that this was because Columbia had licensed to another company. Nonetheless, based on statements by Illumina witness Dr. Barnes and Illumina's own marketing literature, the invention recognized by Illumina as having merit is one which is described in Tsien with the removable 3-'OH capping group and base label. There is insufficient evidence that Illumina's licensing strategy was driven by recognition of the merits of the claimed invention, rather than knowledge of a patent potentially covering their own product.

D. Praise and skepticism

We have considered Columbia's evidence of praise and skepticism, but find it of insufficient weight and relevance to deem it persuasive as to

the merits of the claimed invention particularly when we consider it within the totality of the evidence before us.

E. Summary

After considering the evidence of record, including the secondary considerations, we are persuaded that a preponderance of the evidence supports Illumina's contention that claims 1 and 15 are unpatentable over I) Tsien and Prober I; II) Tsien, Prober I, and Rabani; and III) Tsien and Seela I. Columbia made no substantive arguments that would differentiate claims 2-7, 11, 12, 14, and 17 from claims 1 and 1. These claims are therefore unpatentable for the same reasons as claims 1 and 15, and the reasons set forth in the Petition.

VI. DOWER

In the Decision on the Request for Rehearing, the Board authorized the patentability challenge to claims 1-7, 11, 12, 14, 15, and 17 based Dower as anticipatory publication (Dec. Reh'g 7). Upon reconsideration and in view of Columbia's Response under § 42.120 (Paper 69) and the Trainor Declaration, we shall not sustain this challenge.

Dower describes a DNA sequencing method which uses base-labeled nucleotides (col. 18, l. 64 to col. 19, l. 10) and a reversible blocking agent on the 3'-OH of the nucleotide sugar to allow for deblocking and subsequent elongation (col. 14, ll. 50-53; col. 15, ll. 33-35, 38-40, and 52-56) (Petition 34).

Dower was not said by Illumina to expressly describe a deazapurine base. Rather, Illumina contends in the petition that a nucleotide comprising a deazapurine base is present by virtue of the incorporation by reference of

the Prober I publication which is said to disclose nucleotides with deazapurine bases (Request Reh'g 2-3). The issue addressed in the Request for Rehearing with respect Dower was whether Illumina met its burden in establishing whether Prober I is incorporated into the host document in a manner that complies with the requirement of 35 U.S.C. § 102. The following three passages were cited in the Request for Rehearing to support this determination:

> (c) An alternative polymer stepwise synthetic strategy can be employed. In this embodiment, the fluorophores need not be removable and may be attached to irreversible chain terminators. Examples of such compounds for use in sequencing DNA include, but are not limited to, dideoxynucleotide triphosphate analogs as described by Prober et al. (1987) *Science* 238:336-341.

(Dower, col. 25, ll. 41-47.)

> DNA polymerase, or a similar polymerase, is used to extend the chains by one base by incubation in the presence of dNTP analogs which function as both chain terminators and fluorescent labels. This is done in a one-step process where each of the four dNTP analogs is identified by a distinct dye, such as described in Prober et al. *Science* 238:336-341 . . . .

(*Id.* at col. 23, ll. 18-24.)

> Fluorescent chain terminators (analogs of dATP, dCTP, dGTP, and TP, each labeled with fluorophore preferably emitting at a distinguishable wavelength) are added to the reaction at a sufficient concentration and under suitable reaction conditions (time, temperature, pH, ionic species, etc., See Sambrook et al. (1989) *Molecular Cloning*, vols. 1-3, and Prober et al.) . . . .

(*Id.* at col. 25, ll. 4-10.)

Advanced Display Sys., Inc. v. Kent State Univ., 212 F.3d 1272 (Fed. Cir. 2000), set forth the test for anticipation when material is incorporated by reference. "Incorporation by reference provides a method for integrating

material from various documents into a host document . . . by citing such material in a manner that makes clear that the material is effectively part of the host document as if it were explicitly contained therein." *Id.* at 1282 (citations omitted). "To incorporate material by reference, the host document must identify with detailed particularity what specific material it incorporates and clearly indicate where that material is found in the various documents." *Id.* (citations omitted).

In this case, the passage at column 25, lines 41-47, refers to Prober I in the context of using nucleotides which are "irreversible chain terminators." An irreversible chain terminator is a termination dideoxynucleotide which lacks the removable 3'-OH group (Ex. 2033, Trainor Decl. ¶¶ 24 and 29). In contrast, the claims require a removable blocking group at the 3'-OH group. Thus, this reference to Prober I does not, when combined with Tsien, describe a nucleotide with a "removable chemical moiety capping the 3'-OH group of the sugar" as required by the claim.

The passage at column 23, lines 18-24, refers to chain terminators in reference to Prober I which only describes irreversible chain terminator. In this passage, Prober I is referenced for its teaching of identifying "each of the four dNTP analogs . . . by a distinct dye, such as described in" Prober I (Dower col. 23, ll. 18-24). It therefore appears that Prober I is cited for its disclosure of the concept of using a distinct dye for each nucleotide, and not necessarily for using a deaza-substituted base attached to the dye (Ex. 2033, Trainor Decl. ¶ 55).

In the passage at column 25, lines 4-10, Dower refers to fluorescent chain terminators as being used in sufficient concentrations and suitable

reaction conditions, and then references Sambrook and Prober I. Dr. Weinstock testified that in this passage Dower "expressly teaches the combination with Prober I to make the labeled nucleotides." (Ex. 1021, Weinstock Decl. ¶ 70.) However, this passage refers to analogs of dATP, dCTP, dGTP, and TP which are not the same as the irreversible chain terminators in Prober I. The passage also refers to concentrations and condition to carry out reactions. Therefore, it is not clear from the sentence what Prober I is being cited for and the passage does not state "with detailed particularity what specific material it incorporates and clearly indicate where that material is found" as required under *Advanced Display*.

Accordingly, we find that Illumina has not established by a preponderance of the evidence that Dower anticipates claims 1 and 11, and dependent claims 2-7, 12, 14, 15, and 17.

MOTIONS

VII. COLUMBIA'S MOTION TO AMEND

A motion to amend the claims under 37 C.F.R. § 42.121 was filed by Columbia on August 30, 2013 (Paper 70). In the motion, Columbia proposed: 1) cancelling claim 1; 2) cancelling claims 2-7 and replacing them with claims 19-24; 3) canceling claims 11 and 12 and replacing them with claims 25 and 26; 4) canceling claims 14, 16 and 17 and replacing them with claims 27-29, and 5) cancelling claim 15 and replacing it with claim 18.

Proposed claim 18 is identical to original claim 15, rewritten in independent form and reciting all the features of original claim 1.

Proposed claims 19-23 are identical to original claims 2-6, respectively, except they depend from proposed claim 18.

Proposed claim 24 is identical to original claim 7, except that it depends from proposed claim 23.

Proposed claims 25 is identical to original claim 11 except that it specifies a plurality of different nucleic acid templates. Proposed claim 26 is identical to claim 12, except that it depends on proposed claim 25.

Proposed claims 27-29 are identical to claims 14, 16, and 17.

The main differences between the original claims and the proposed claims are that 1) claim 1 has been amended by incorporating the limitation of claim 15 requiring that "each of said unique labels is attached to the nucleotide analogue via a cleavable linker," and designating it as proposed claim 18; and 2) claim 11 has been amended by adding the limitation that the recited nucleic acid templates are "different," and designating it as proposed claim 25. The latter limitation was not in original claim 11 or in any of the original claims, which depended on claim 11. However, Columbia cited support in the '698 Patent for the limitation (Paper 70, p. 8-9).

Claim 25 adds the limitation "different" to claim 11, but the claim is otherwise identical to claim 11. Columbia, in adding this term, did not give a reason as to why the proposed claim is patentably distinct over the prior art or how it responds to a ground of unpatentability under 37 C.F.R. § 42.121(a)(2)(i). Indeed, Columbia stated that the amendment "merely clarifies the original patent claim, as the original patent claim would have been understood by a person skilled in the art" because a person of ordinary skill in the art would have understood original claim 11 to mean different nucleic acid templates (Paper 70, p. 13). Thus, by Columbia's own admission, claims 25 is of the same scope as original claim 11.

Accordingly, all the claims proposed in the Columbia amendment are of the same scope as claims already before us in this review, and which have been determined to be unpatentable. In the opposition to the motion, Illumina contends that Columbia's motion is defective (Paper 74, p. 1). We need not, and do not, reach Illumina's contention, however, since the claims, even as Columbia proposes to amend them, are unpatentable.

VIII. COLUMBIA'S MOTION TO EXCLUDE

A motion to exclude evidence under 37 C.F.R. § 42.64 was filed by Columbia on November 12, 2013 (Paper 93).

A. Columbia seeks to exclude Exhibits 1029-1033, which were said to have been introduced for the first time at the deposition of Illumina's expert, Dr. Weinstock, during redirect examination by Illumina's counsel (Paper 93, p. 1). As we did not rely on this portion of Dr. Weinstock's testimony, or the exhibits cited in it, we dismiss this part of the motion as moot.

B. Columbia seeks to exclude Exhibits 1041-1049, which were introduced at Dr. Trainor's deposition (Paper 93, p. 4). Exhibits 1041-1048 were introduced by Illumina for the purpose of impeaching Dr. Trainor's opinions in his declaration regarding the non-obviousness of the claimed subject matter (Ex. 2094, Trainor Tr. 277: 21 to 278: 6). Columbia contends that these references were belatedly introduced so that they could be cited in Illumina's Reply and in Exhibit 1053 (Declaration of Kevin Burgess, Ph.D.) in order to make out Illumina's prima facie case, in violation of the Trial Practice Guide (77 Fed. Reg. 48756, 48767 (Aug. 14, 2012)). (Paper 93, p. 6). Exhibit 1049 is a declaration from an *inter partes* review to which

Columbia is not a party, previously introduced as Ex. 1024, which was previously expunged by the Board as improperly filed (Paper 34).

We have determined there was a reason to have made the claimed nucleotides based on the combination of Tsien and Prober I without relying on Exhibits 1041-1049. Thus, we dismiss this part of the motion as moot.

C. Columbia seeks to exclude Exhibits 1050-1054 (Paper 93, p. 7). Exhibits 1050, 1051, 1052, and 1054 are said by Columbia belatedly to raise new issues and evidence to make out its prima facie case (*id*.). Exhibit 1053 is a declaration of Kevin Burgess filed by Illumina and cited for the first time in their response to Columbia's response under § 42.120 (Paper 76, Pet'r Reply 2).

We determine that the claims are unpatentable without relying on Exhibits 1050-1054 and thus we dismiss this portion the motion as moot as well.

IX. ILLUMINA'S MOTION TO EXCLUDE

A motion to exclude evidence was filed by Illumina on November 12, 2013 (Paper 90). This evidence goes to the secondary considerations that were argued by Columbia in their response to the Petition under § 42.120. As we conclude that the Columbia claims are unpatentable even if we consider this evidence, we need not and do not decide this motion and dismiss it as moot.

X. ORDER

In consideration of the foregoing, it is

ORDERED that claims 1-7, 11, 12, 14, 15, and 17 of U.S. Patent 7,713,698 B2 are cancelled;

FURTHER ORDERED that Columbia's motion to amend claims is denied;

FURTHER ORDERED that Columbia's motion to exclude evidence is dismissed as moot; and

FURTHER ORDERED that Illumina's motion to exclude evidence is dismissed as moot.

Petitioner:

Robert Lawler

And

James Morrow
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Patent Owner:

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Paper 43
Mailed: May 10, 2013

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE PATENT TRIAL AND APPEAL BOARD

_____

ILLUMINA, INC.
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner.

_____

Case IPR2012-00006
U.S. Patent 7,713,698

_____

Before SALLY G. LANE, RICHARD M. LEBOVITZ, and DEBORAH KATZ, *Administrative Patent Judges*.

LEBOVITZ, *Administrative Patent Judge*.

DECISION ON REQUEST FOR REHEARING UNDER 37 C.F.R. § 41.71(c) OF DECISION TO INSTITUTE INTER PARTES REVIEW & SCHEDULING ORDER

I. REHEARING REQUEST

Illumina requests rehearing of the decision under 35 U.S.C. § 311 ("the '698 Decision") (Paper 28) denying inter partes review of claims 15 and 16 of US 7,713,698 ("the '698 patent") based on Tsien and Dower as anticipatory publications (Illumina Request for Reconsideration under 37 C.F.R. 42.71(c), dated March 26, 2013 ("Illumina Req. Reh'g."; Paper 30))

Under 37 C.F.R. § 42.71(c), "[w]hen rehearing a decision on petition, a panel will review the decision for an abuse of discretion." An abuse of discretion occurs when a "decision was based on an erroneous conclusion of law or clearly erroneous factual findings, or . . . a clear error of judgment." *PPG Indus. Inc. v. Celanese Polymer Specialties Co. Inc.*, 840 F.2d 1565, 1567 (Fed. Cir. 1988). *See also* 37 C.F.R. § 42.71(d) ("The request must specifically identify all matters the party believes the Board misapprehended or overlooked").

I.A. THE BOARD ERRED IN NOT AUTHORIZING INTER PARTES REVIEW OF CLAIMS 1 AND 11 BASED ON TSIEN & DOWER (ILLUMINA REQ. REH'G 3)

Nucleotides comprise a sugar, phosphate, and nitrogen base ('698 patent, Fig. 7). Claim 1 is drawn to nucleic acid sequencing method comprising employing at least one nucleotide which comprises a deazapurine as the nitrogen base. Claim 11 is drawn to a primer hybridized to a nucleic acid template, where at least one of the nucleotides in the primer comprises a deazapurine as a base. Neither Tsien nor Dower is said by Illumina to expressly describe a deazapurine base in their written disclosures. Rather, Illumina contends the nucleotides are present by virtue

of the incorporation by reference to the Prober I publication by Tsien and Dower. Dower is said to disclose nucleotides with deazapurine bases (Petition 26 & 39-40). The issue addressed in the '698 Decision with respect to these rejections was whether Illumina met its burden in establishing whether Prober I is incorporated into the host document in a manner that complies with the requirement of 35 U.S.C. § 102 ('698 Decision 10-11).

"To incorporate material by reference, the host document must identify with detailed particularity what specific material it incorporates and clearly indicate where that material is found in the various documents." *Advanced Display Sys., Inc. v. Kent State Univ.*, 212 F.3d 1272, 1282 (Fed. Cir. 2000). When making such determination, the standard "of one reasonably skilled in the art should be" applied. *Id.* at 1283. Illumina contends that we erred by not using "the person of reasonable skill in the art" standard to evaluate whether Tsien (Exhibit 1002) and Dower (Exhibit 1005) incorporated Prober I's (Exhibit 1003) disclosure of a 7-deazapurine base (Illumina Req. Reh'g 4). Specifically, Illumina contends that we "used a heightened standard that would require recitation of the exact word 'deazapurine' (or at least 'deaza-substituted') as used in claims 1 and 11" (*id.*). To support their argument, Illumina points to declarations by Dr. Weinstock and Dr. Blanchaud (*id.* at 5).

I.A.1. Dower and Prober I

We agree with Illumina that we erred in not instituting inter partes review of claims 1 and 11 based on Dower as an anticipatory publication.

On page 12 of the '698 Decision, we reproduced the following passage of Dower which referred to Prober I:

> (c) An alternative polymer stepwise synthetic strategy can be employed. In this embodiment, the fluorophores need not be removable and may be attached to irreversible chain terminators. Examples of such compounds for use in sequencing DNA include, but are not limited to, dideoxynucleotide triphosphate analogs as described by Prober et al. (1987) Science 238:336-341.

Dower, col. 25, ll. 41-47.

On pages 33 and 34-35 of the Petition, Illumina referred to the following additional disclosure:

> DNA polymerase, or a similar polymerase, is used to extend the chains by one base by incubation in the presence of dNTP analogs which function as both chain terminators and fluorescent labels. This is done in a one-step process where each of the four dNTP analogs is identified by a distinct dye, such as described in Prober et al. *Science* 238:336-341

Dower, col. 23, ll. 18-24.

> Fluorescent chain terminators (analogs of dATP, dCTP, dGTP, and TP, each labeled with fluorophore preferably emitting at a distinguishable wavelength) are added to the reaction at a sufficient concentration and under suitable reaction conditions (time, temperature, pH, ionic species, etc., See Sambrook et al. (1989) Molecular Cloning, vols. 1-3, and Prober et al.).

Dower, col. 25, ll. 4-10.

It is evident from the above quoted disclosure from Dower that Dower is referencing Prober I for all its dNTP analogues, at least one of which is a deazapurine. For example, Dower, at column 23, lines 18-24 and column 25, lines 41-47, refers to Prober I's disclosure of nucleotide analogs (dNTP and dideoxynucleotide triphosphate) in DNA sequencing.

To support the position that such disclosure is an adequate incorporation by reference for the purpose of anticipation, Illumina cites *Callaway Golf Co. v. Acushnet Co.*, 576 F.3d 1331 (Fed. Cir. 2009) (Illumina Req. Reh'g 8-9, 10, and 12). Illumina contends that *Callaway* holds that "reference **to a general class of compositions** discussed in prior art is **sufficient to incorporate a specific composition** by reference. *Callaway*, 576 F.3d at 1346-47 (holding reference to a 'foamable polymeric composition' sufficient to specifically incorporate polyurethane as the foamable composition)" (*id.* at 10-11). We agree that the holding in Callaway is relevant in the circumstances before us.

In *Calloway*, the Federal Circuit wrote:

> Nesbitt [the cited anticipatory publication] states broadly that the layers of the golf ball disclosed therein may be made from a "natural or synthetic polymeric material." [U.S. Patent No. 4,431,193] col. 3 ll. 53-54. Nesbitt goes on to directly indicate that such materials include all of the foamable polymeric materials described in Molitor [U.S. Patent No. 4,274,637]: "Reference is made to [Molitor ″637] which describes a number of foamable compositions of a character which may be employed for one or both layers … for the golf ball of this invention." *Id*. col.3 ll. 56–61 (emphasis added). Polyurethane is a foamable composition. Nesbitt incorporates the entire list of foamable compounds ("a number of foamable compositions") disclosed by Molitor ″637 as appropriate materials for use in golf ball cover layers, including polyurethane and mixtures of ionomer resins.

Callaway, 576 F.3d at 1347-48.

Dower incorporates all the nucleotide analogues from Prober I, which includes an analogue comprising a deazapurine base. Consistent with *Callaway*, the incorporation by reference to Prober I in Dower is therefore sufficient to establish that Dower describes within its four corners a method

of using a nucleotide comprising a deazapurine base as required by claims 1 and 11.

Columbia in their opposition to Illumina's Rehearing Request argues that Dower refers to Prober I six times, "but not once uses the words 'incorporated herein by reference' whereas Dower refers to other specific references seventeen times using the words 'incorporated herein by reference." (Columbia's Opposition 2, dated April 26, 2013; Paper 37.) Columbia has not cited any legal authority that the words "incorporated herein by reference" are necessary to incorporate Prober I's disclosure into Dower. Rather, we find that Dower's reference to Prober I for dNTP analogs in DNA sequencing is sufficient for a person of reasonable skill in the art to have recognized that Prober I is incorporated for this teaching.

Columbia also notes that the Board relied upon the disclosure at column 25, lines 41-47 of Dower in the '698 Decision, but that this passage was not cited by Illumina in their petition (Columbia Opposition 3). We acknowledge this fact to be true, but the disclosure appears on the same column 25 (at lines 4-10 and lines 35-37) cited by Illumina in their petition (Petition 35, 37, 39, and 41), and thus our attention was clearly drawn to column 25 for its pertinence. In addition, the passage at column 25, lines 4-10 expressly cited by Illumina refers to "[f]luorescent chain **terminators**" and the passage at column 25, lines 41-47 appears in the section titled "There are several suitable labeled, **terminator** structures as follows." Thus it was reasonable to consider the disclosure at column 25, lines 41-47 based on the passage expressly cited by Illumina in their petition.

Columbia further argues that even if Dower had incorporated by reference the deazapurines disclosed in Prober I, the '698 Decision "that

Dower did not anticipate the claims at issue would still be correct. The deaza-substituted dideoxynucleotides of Prober I do not contain a removable chemical moiety capping the 3'-OH group of the sugar as required by the claims." (Columbia Opposition 5, n.4).

Illumina in their petition points to specific passages of Dower which describe modified analogs which "should" be blocked at the 3'-end of elongation (Petition 35). Columbia has not identified a defect in this specific disclosure which teaches utilizing 3'-blocked analogs in DNA sequencing, and we find none.

For the foregoing reasons, we **authorize** an inter partes review of claims 1 and 11 instituted with respect to Illumina's patentability challenge based on Dower.

Claims 2-7, 11, 12, 14, 15, and 17

With respect to dependent claim 2-7, 11, 12, 14, 15, and 17, Illumina identified specific disclosure in Dower where each limitation is found (Petition 35-39). We find Illumina's factual assertions to be supported, and authorize an inter partes review of claims 2-7, 11, 12, 14, 15, and 17 to be instituted with respect to Illumina's patentability challenge based on Dower as an anticipatory publication.

I.A.2. Tsien & Prober I

Contrary to Illumina's contention, the '698 Decision did not ignore how a person of reasonable skill in the art would have viewed Tsien's incorporation by reference to Prober I. The only evidence of how such a

person would have understood the reference to Prober I in Tsien was the declaration of Dr. George Weinstock.

> Dr. Weinstock stated in his declaration (Exhibit 1021):
>
> > In discussing sequencing by synthesis methods utilizing a dNTP in which the fluorescent label group is coupled to the base of the dNTP, Tsien references the disclosure of Prober I, *Science* 238, 336-341 (1987) for its teaching of preparing nucleotides with fluorescent tags that can be successfully incorporated by Tsien's preferred polymerase. See Tsien et al., page 5, lines 22- 23, page 19, lines 4-18; and page 28, lines 5-18. Tsien also states that Prober I, and the other references discuss in Tsien, are referenced "for their teaching of synthetic methods, coupling and detection methodologies, and the like." Tsien, p. 3, ll. 11-16 and p. 5, ll. 22-23.
>
> (Weinstock Decl. ¶ 63.)

Based on the cited disclosure in Tsien, Dr. Weinstock concluded that he understood "Tsien to incorporate the teachings of Prober I for its teachings regarding of fluorescent label attachment, and in particular, regarding its teaching regarding attachment of a linker to the 7 position in the 7-deazapurine" (*id.* at ¶ 63).

The '698 Decision did not find Dr. Weinstock's testimony persuasive because "Dr. Weinstock did not provide a factual basis for his testimony as to why this teaching would have been recognized by one of ordinary skill in the art." ('698 Decision 13.) In particular, it was noted in the Decision that "Illumina provided testimony by Dr. George Weinstock that Prober I is incorporated by Tsien for 'its teaching regarding attachment of a linker to the 7 position in the 7-deazapurine,' but these words do not appear in Tsien. Weinstock Decl. ¶ 63." (*Id.* at 13.) Dr. Weinstock's testimony was determined to be inadequate to make up for this deficiency.

Tsien does not indicate that Prober I is being referenced for its teaching of a deazapurine nucleotide. Tsien refers to Prober I for its teaching that fluorescent ddNTPs can be incorporated by two different enzymes, but did not identify the fluorescent ddNTPs as comprising a deazapurine base as recited in claims 1 and 11 ('698 Decision 11-12). Dr. Weinstock's testimony did not bridge the gap. Thus, while the Decision addressed Tsien's failure to specifically identify the deazapurines in Prober I, the Decision found the incorporation defective for the purpose of establishing anticipation because Dr. Weinstock's testimony did not sufficiently explain why the reasonably skilled person would have understood the reference to Prober I to be a reference to deazapurines.

In the Request for Rehearing, Illumina attempts to remedy this deficiency, stating "[s]ignificantly, the only analogs of dATP and dGTP present in the Prober I reference are 7-deazapurines" and explains why this is the case (Illumina Req. Reh'g 6). However, as pointedly stated by Illumina, the determination of "incorporation by reference" for the purpose of establishing anticipation is from the viewpoint of one reasonably skilled in the art. The identification of where Prober I described deazapurines, and the explanation of why Tsien's disclosure "fluorescent ddNTPs" would be understood to be these deazapurines was not discussed in Dr. Weinstock's declaration, but rather is only described in this Request for Rehearing signed by Illumina's attorney. The argument of counsel cannot take the place of evidence lacking in the record. *Estee Lauder Inc. v. L'Oreal, S.A.*, 129 F.3d 588, 595 (Fed. Cir. 1997).

Moreover, 37 C.F.R. § 41.71(d) states a Request for Rehearing "must specifically identify . . . the place where each matter was previously

addressed in a motion, an opposition, or a reply." Illumina has not cited where in the '698 Petition it was argued that the only analogs of dATP and dGTP described in Prober I are 7-deazapurines.

Blanchaud Declaration

Illumina also states that "Blanchaud understood certain features to be included in the incorporation of Prober based on the general description of the incorporated matter in Tsien and Dower, respectively." (Illumina Req. Reh'g 5.) In fact, Blanchaud only appears in the list of references cited on pages v-vi of the Petition, but was not mentioned in any other place in the Petition. Accordingly, we did not address Blanchaud in the Decision. It is therefore not a matter misapprehended or overlooked by the Board as required by 37 C.F.R. § 42.71(d).

Calloway

Unlike in *Calloway* (reproduced above), Tsien did not disclose that all the materials in Prober I are to be included in their sequencing methods. For example, Tsien does not refer to Prober I for disclosing fluorescent nucleotides that could be used in its method, but rather referred to Prober I for "show[ing] enzymatic incorporation of fluorescent ddNTPs" using two different enzymes ('698 Decision 25). Illumina did not direct us to adequate factual evidence or testimony by one reasonably skilled in the art that such sentence serves to incorporate the specific ddNTPs described in Prober I.

I.B. DUPLICATIVE REJECTIONS

Citing 37 C.F.R. § 42.108, we denied certain unpatentability challenges to the claims. Illumina contends that "the Board abused its discretion by considering an improper factor when deciding whether to authorize *inter partes* review." (Req. Reh'g 13.) Illumina contends that the Board's "discretion is limited to determining whether, in fact, the references render the challenged patent unpatentable. The Patent Rules do not authorize the Board to exercise discretion based on whether or not certain references are cumulative or duplicative of each other." (*Id.*at 14.)

Under 37 C.F.R. § 42.108(a), the Board has discretion to "authorize the review to proceed on all or some of the challenged claims and on all or some of the grounds of unpatentability asserted for each claim." The Board also "may deny some or all grounds for unpatentability for some or all of the challenged claims." 37 C.F.R. § 42.108(b). As stated in the '698 Decision, in making such determinations, the Board must also take into account 37 C.F.R § 42.1(b) which requires "the just, speedy, and inexpensive resolution of every proceeding."

In this case, the decision not to authorize inter partes review on certain unpatentability challenges was based on the finding that the challenges appeared to rely on the same prior art facts as other challenges for which inter partes review had been authorized. The determination not to proceed on all of the proposed unpatentability challenges by Illumina was therefore grounded on the determination that the same facts were being applied to the claims, albeit using different publications to establish that a fact was prior art to the claims. The concern was that the redundant unpatentability challenges would impede "the just, speedy, and inexpensive resolution of every

proceeding" as required under 37 C.F.R § 42.1(b). In other words, considering multiple rejections for the same unpatentability issue would unnecessarily consume the time and resources of all parties involved. As Illumina did not provide a meaningful distinction between the different, redundant rejections, we perceived no unfairness by not authorizing what appeared to be redundant challenges because an inter partes review had been instituted on the same factual basis.

Illumina contends:

> [A]lthough the references are used to support invalidity contentions regarding the same claims, the references themselves are not identical. Thus, the Patent Owner may assert that a claim element is not present, or would not be obvious to combine with another reference, when that same element is more clearly set forth in a different reference.

(Req. Reh'g 14.)

While it is true that the cited references are not identical, the cited references appear to have been cited for the same facts. Illumina speculates that in certain publications an element may be more clearly set forth in one publication rather than another, but has not provided a persuasive example of such a case.

Dower

Illumina contends that Dower is not duplicative to Tsien, and cites one such difference (Req. Reh'g 14-15). However, Illumina did not provide an adequate explanation as to how this difference would impact the unpatentability challenge.

II. SCHEDULING ORDER

In the conference call on April 16, 2013 between Illumina, Columbia, and the PTAB panel, Columbia requested a change in the Scheduling Order (Paper 29) to provide more time, depending on the outcome of the decision on Illumina's Request for Rehearing. While the '698 Decision on Petition has been modified, the modification is not deemed sufficient to warrant a time extension on any of the dates in the Scheduling Order. Columbia's request is therefore DENIED.

III. SUMMARY

The '698 Decision is modified as follows:

Pursuant to 35 U.S.C. § 314, in addition to challenges I-III identified in the '698 Decision, we authorize inter partes review to be instituted as follows:

IV. Claim 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 102(b) as anticipated by Dower.

GRANTED-IN-PART

Case IPR2012-00006
U.S. Patent 7,713,698

Petitioner:

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Paper 28
Mailed: March 12, 2013

UNITED STATES PATENT AND TRADEMARK OFFICE

_____

BEFORE THE PATENT TRIAL AND APPEAL BOARD

_____

ILLUMINA, INC.
Petitioner,

v.

THE TRUSTEES OF COLUMBIA UNIVERSITY IN THE CITY OF NEW YORK
Patent Owner.

_____

Case IPR2012-00006
U.S. Patent 7,713,698

_____

Before SALLY G. LANE, RICHARD M. LEBOVITZ, and DEBORAH KATZ, *Administrative Patent Judges*.

LEBOVITZ, *Administrative Patent Judge*.

DECISION ON PETITION FOR

INTER PARTES REVIEW UNDER 35 U.S.C. § 311

This is a decision on a petition[1] under 35 U.S.C. § 311 challenging the patentability under 35 U.S.C. §§ 102 and 103 of claims 1-7, 11, 12, 14, 15, and 17 of U.S. Pat. 7,713,698 B2. We have jurisdiction to decide the petition under 37 C.F.R. §§ 42.3(a) and 42.4(a). Pursuant to 35 U.S.C. § 314, we authorize inter partes review to be instituted on all of the challenged claims as follows:

I. Claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien[2] and Prober I.[3] Petition 27.

II. Claims 5 and 12 under 35 U.S.C. § 103(a) as obvious in view of Tsien, Prober I, and Rabani.[4] Petition 52.

III. Claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien and Seela I.[5] Petition 56.

STATEMENT OF THE CASE

Illumina petitions under 35 U.S.C. § 311 to institute inter partes review of U.S. Patent 7,713,698 ("the '698 patent"). Petition 2. In their petition under 35 U.S.C. § 312, Illumina identifies 22 different grounds challenging the patentability under 35 U.S.C. §§ 102 and 103 of claims 1-7, 11, 12, 14, 15, and 17 of the '698 Patent. *Id.* at 5-8. Based on the prior art cited in each ground, Illumina contends that claims 1-7, 11-12, 14-15 and 17

[1] Petition for Inter Partes Review of U.S. Pat. No. 7,713,698. Paper 3 (September 16, 2012).
[2] Roger Tsien et al., WO 91/06678 (May 16, 1991), Exhibit 1002.
[3] James Prober et al., A System for Rapid DNA Sequencing with Fluorescent Chain-Terminating Dideoxynucleotides, 238 Science 336 (1987), Exhibit 1003.
[4] Ely Rabani et al., WO 96/27025 (September 6, 1996), Exhibit 1006.
[5] Frank Seela, U.S. 4,804,748 (February 14, 1989), Exhibit 1014.

of the '698 patent are invalid. The Patent Owner of the '698 patent is The Trustees of Columbia University in the City of New York ("Columbia"). Columbia filed a Preliminary Response under 37 C.F.R. § 42.107 to Illumina's patentability challenge.[6]

The '698 Patent

The '698 patent issued May 11, 2010. The named inventors are Jingyue Ju, Zengmin Li, John Robert Edwards, and Yasuhiro Itagaki. The invention of the '698 patent involves sequencing DNA by incorporating a nucleotide analogue into a newly synthesized strand of DNA, and then detecting the identity of the incorporated analogue. A polymerase is used to incorporate the nucleotide analogue into the strand of DNA. '698 Patent, col. 2, ll. 24-28. The method is generally referred to as "sequencing DNA by synthesis" because the *sequence* of the DNA is determined by identifying the successive additions of labeled nucleotides to a newly *synthesized* strand of DNA. *Id.* at col. 2, ll. 6-11. The sequence is determined as the DNA is synthesized – using a complimentary DNA strand as a template to direct DNA synthesis.

The '698 patent is the subject of the litigation styled *The Trustees of Columbia University in the City of New York v. Illumina, Inc.*, 1:12-cv-00376-UNA, currently pending in the United States District Court for the District of Delaware. Petition 3-4. According to Illumina, Columbia alleges in that litigation that Illumina has infringed and continues to infringe the '698 patent. *Id.*

[6] Columbia's Preliminary Response under 37 C.F.R. § 42.107. Paper 21 (December 20, 2012).

Related petitions for inter partes review

There are two pending inter partes petitions which are related to this petition for inter partes review in the '698 patent.

A petition for inter partes review was filed on September 16, 2012 for U.S. Pat. No. 7,790,869 B2 ("the '869 patent").[7] The '869 patent has the same lineage as the '698 patent, including tracing its ancestry back to a continuation-in-part of application of Appl. No. 09/684,670, filed October 6, 2000 and a provisional application filed June 26, 2001.

A petition for inter partes review was filed on October 3, 2012 for U.S. Pat. No. 8,088,575 B2 ("the '575 patent")[8] which is based on a continuation application of the '869 patent.

All three petitions have been decided concurrently.

The claims

The '698 patent was granted with 17 claims. Illumina challenges the validity of independent claims 1 and 11, and dependent claims 2-7, 12, 14, 15, and 17. Claims 1 and 11 are reproduced below (emphasis added):

> 1. A method of determining the identity of a nucleotide analogue incorporated into a nucleic acid primer extension strand, comprising:
>
> a) contacting a nucleic acid template attached to a solid surface with a nucleic acid primer which hybridizes to the template;
>
> b) simultaneously contacting the product of step a) with a polymerase and four nucleotide analogues which are either (i) aA, aC, aG, and aT, or (ii) aA, aC, aG, and aU, so as to incorporate one of the nucleotide analogues onto the nucleic

[7] IPR2012-00007.
[8] IPR2013-00011.

> acid primer and form a nucleic acid primer extension strand, wherein each nucleotide analogue within (i) or (ii) comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the nucleotide analogue, and *wherein at least one of the four nucleotide analogues within (i) or (ii) is deaza-substituted*; and
>
> c) detecting the unique label of the incorporated nucleotide analogue,
>
> so as to thereby determine the identity of the nucleotide analogue incorporated into the nucleic acid primer extension strand.
>
> 11. A plurality of nucleic acid templates immobilized on a solid surface, wherein a nucleic acid primer is hybridized to such nucleic acid templates each such nucleic acid primer comprising a labeled incorporated nucleotide analogue, at least one of which is deaza-substituted, wherein each labeled nucleotide analogue comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the nucleotide analogue.

CLAIM INTERPRETATION

Both independent claims 1 and 11 recite that at least one of the nucleotide analogues is "deaza-substituted." One of ordinary skill in the art would understand that a "deaza-substituted nucleotide" is a nucleotide analogue which includes a deazabase as the nitrogen base (e.g., adenine, guanine, cytosine, or thymine). '698 patent, col. 7, ll. 44-63. A deazabase is a nitrogen base in which one of the natural nitrogen atoms in the base ring is substituted with a carbon atom. *Id.* For example, in a 7-deazapurine, the natural 7-position nitrogen in the base ring is replaced with a carbon atom. *Id.*

Claim 1 comprises a step in which a "nucleic acid template attached to a solid surface" is contacted with a nucleic acid primer. Claim 11 recites that a "plurality of nucleic acid templates immobilized on a solid surface." The meaning of the terms "attached to" and "immobilize on" solid surfaces is in dispute. We thus begin with claim interpretation because before a claim is properly interpreted, it cannot be compared to the prior art.

During inter partes review claims are given their broadest reasonable interpretation in view of the specification of which they are part. 37 C.F.R. § 42.100(b); see Office Patent Trial Practice Guide, 77 Fed. Reg. 48756, 48766 (Aug. 14, 2012). Accordingly, we first turn to the '698 patent disclosure.

Neither of the contested phrases ("attached" (claim 1) or "immobilized" (claim 11) to a solid surface) are expressly defined in the '698 Patent. However, there is guidance in the patent as to their meaning.

In the "Background of the Invention," the inventors describe prior art methods for sequencing DNA, particularly "sequencing DNA by synthesis." The inventors state:

> The present application discloses a novel and advantageous system for DNA sequencing by the synthesis approach which employs a stable *DNA template*, which is able to self prime for the polymerase reaction, *covalently linked* to a solid surface such as a chip, and 4 unique nucleotides analogues.

'698 Patent, col. 4, ll. 6-10 (emphasis added).

The '698 Patent does not use the term "covalently linked to a solid surface" again to characterize the attachment of the nucleic acid template to a solid surface. However, in the "Summary of the Invention," the only disclosed method of attaching the nucleic acid to a surface involves a chemical "interaction" which is "between the phosphine moiety [coated] on

the solid surface and the azido group on the 5' end of the nucleic acid" which serves to "immobiliz[e] the 5' end of the DNA to the solid surface." '698 Patent, col. 5, ll. 3-11. This interaction between the phosphine and azido group is described as enabling "specific coupling of the DNA template with the solid surface." *Id.* at col. 3, l. 61 to col. 4, l. 2; col. 23, ll. 59-67. Figure 3 shows a covalent linkage between the phosphine and azido groups. *See id.* at Fig. 3; col. 5, ll. 47-50. This method is the only specifically described method disclosed in the Summary of the Invention, in the Detailed Description of the Invention (*id.* at col. 12, ll. 5-13), and in the Examples *(id.* at col. 23, ll. 59-67; col. 29, ll. 1-10) – although there appears to be more general disclosure elsewhere in the patent (*id.* at col. 22, ll. 16-18). Finally, the '698 Patent describes a DNA template strand "directly attached to the solid surface." *Id.* at col. 9, ll. 28-30.

Based on this description, we interpret the terms "attach" and "immobilize" to mean the same thing, since both are used interchangeably in the '698 Patent. '698 Patent, col. 5, ll. 3-11; col. 12, ll. 5-13. The terms clearly encompass covalent attachment in which the DNA is covalently attached to the surface through a chemical linkage since such attachment is specifically and repeatedly exemplified in the '698 Patent. *Id.* at col. 4, ll. 6-10; col. 5, ll. 47-50; Fig. 3. However, we cannot discern any language in the claim or patent specification which would require us to limit the claims to covalent linkage.

Nonetheless, the specific disclosures in the '698 Patent of "covalent linkage" of two chemical moieties between one on the solid surface and one on the DNA" ('698 patent, col. 4, ll. 6-10; Fig. 4), "specific coupling" to the solid surface (*id.* at col. 3, l. 61 to col. 4, l. 2; col. 23, ll. 59-67), and DNA

which is "directly attached" to the surface (*id.* at col. 9, ll. 28-30) imbue the terms "attached" and "immobilized" with a specific meaning that the DNA is joined or fastened directly to the solid surface. In each case, there is no intervening structure or linkage. Rather, the DNA, itself, is fastened to the solid surface, consistent with the ordinary meaning of "attach" to mean "fasten, "join," or "affix."[9] Accordingly, providing the broadest reasonable interpretation when read in light of the '698 patent specification, the terms "attach" and "immobilize" are interpreted to mean that the DNA, itself, is affixed to the solid surface without intervening structures.

1. ANTICIPATION

Independent claim 1 is directed to a "method of determining the identity of a nucleotide analogue incorporated into a nucleic acid primer extension strand," comprising steps of:

a) contacting a nucleic acid template with a nucleic acid primer;

b) simultaneously contacting the product of a) with polymerase and nucleotide analogues of adenine (A), cytosine (C), guanine (G), and thymine (T) or uracil (U), each having a unique detectable label which enables identity of the analogue to be determined in step c), where at least one of the nucleotides is deaza-substituted; and

c) detecting the unique label of the incorporated nucleotide analogue.

Independent claim 11 is drawn to the nucleic acid templates hybridized with the primer (product of step (a) of claim 1), where the primer has incorporated at least one deaza-substituted nucleotide (product of step (b) of claim 1).

[9] http://www.thefreedictionary.com/attach. Accessed January 11, 2013.

Illumina contends that claims 1 and 11, and dependent claims 2-7, 12, 14, 15, and 17, are anticipated under 35 U.S.C. § 102(b) by each of Tsien, Dower,[10] Stemple II,[11] and Stemple III.[12] Petition 12. Under 35 U.S.C. § 102(b), "a person shall be entitled to a patent unless the invention was patented or described in a printed publication . . . more than one year prior to the date of the application." "Accordingly, invalidity by anticipation requires that the four corners of a single, prior art document describe every element of the claimed invention, either expressly or inherently, such that a person of ordinary skill in the art could practice the invention without undue experimentation." *Advanced Display Sys., Inc. v. Kent State Univ.*, 212 F.3d 1272, 1282 (Fed. Cir. 2000).

Illumina, as the moving party, has the burden of proof to demonstrate that there is "a reasonable likelihood that [it] would prevail" in establishing unpatentability of the challenged claims. 35 U.S.C. §§ 311 & 314; 37 C.F.R. § 42.20(c). Thus, Illumina has the burden of establishing that there is reasonable likelihood it would prevail in establishing that the claimed invention of the '698 patent is described within the four corners of each of Tsien, Dower, Stemple II, and Stemple III. We therefore must look to the evidence put forth by Illumina in support of their challenge to the patentability of claims 1-7, 11, 12, 14, 15, and 17.

[10] William Dower et al., U.S. 5,547,839 (August 20, 1996), Exhibit 1005.
[11] Derek Stemple et al., WO 00/53805 (September 14, 2000), Exhibit 1007.
[12] Derek Stemple et al., U.S. 7,270,951 B1 (September 18, 2007), Exhibit 1008.

A. Patentability challenge based on Tsien and Dower (Petition 18 & 30)

Illumina contends that each of Tsien and Dower, describe steps a) through c) recited in claim 1, and the product of claim 11.

Tsien describes a method of sequencing DNA. Tsien, p. 6, ll. 28-30. The method uses labeled nucleotides (dNTP), each with a different detectable label attached to it. *Id.* at p. 7, ll. 3-14; p. 10, ll. 7-10; p. 14, ll. 12-26.

Dower also describes methods for sequencing DNA. Dower, col. 6, ll. 19-20. In one embodiment, a primer is elongated, one nucleotide at a time, using labeled nucleotide analogs in a polymerase-catalyzed enzymatic reaction. *Id.* at col. 14, ll. 37-59; col. 15, l. 56 to col. 16, l. 21. Because this method involves the synthesis of DNA, it is referred to by Dower as the "synthetic method." *Id.* at col. 23, beginning at line 15.

Step b) of claim 1 comprises contacting a nucleic acid template with at least one deaza-substituted nucleotide analogue. Illumina contends that such nucleotides are not expressly disclosed in either the Tsien or Dower disclosures, but are present by virtue of the incorporation into Tsien and Dower of the Prober I publication which is said to disclose deaza-substituted nucleotides. Petition 26.

An anticipatory document must "disclose all elements of the claim within the four corners of the document" and "arranged as in the claim." *Net MoneyIN, Inc. v. VeriSign, Inc.*, 545 F.3d 1359, 1369 (Fed. Cir. 2008). However, "[m]aterial not explicitly contained in the single, prior art document may still be considered for purposes of anticipation if that material is incorporated by reference into the document." *Advanced Display*, 212 F.3d at 1282. Accordingly, the question before us is whether Illumina met

its burden in establishing that Prober I is incorporated into the Tsien and Dower documents in a manner that complies with the requirement of 35 U.S.C. § 102, i.e., that the invention be described within the four corners of the anticipatory reference.

Advanced Display set forth the test for anticipation when material is incorporated by reference. "Incorporation by reference provides a method for integrating material from various documents into a host document … by citing such material in a manner that makes clear that the material is effectively part of the host document as if it were explicitly contained therein." *Advanced Display*, 212 F.3d at 1282 (citations omitted). "To incorporate material by reference, the host document must identify with detailed particularity what specific material it incorporates and clearly indicate where that material is found in the various documents." *Id.* (citations omitted).

The pertinent passages of Tsien and Dower relied upon by Illumina are reproduced below:

Tsien

> One method involves the use of a fluorescent tag attached to the base moiety. . . This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence. Sarfati et aI[.] (1987) demonstrates the incorporation of biotinylated dATP in nick translations, and other biotinylated derivatives such as 5-biotin (19)-dUTP (Calbiochem) are incorporated by polymerases and reverse transcriptase. Prober et al. (1987) show enzymatic incorporation of fluorescent ddNTPs by reverse transcriptase and Sequenase™[.]

Tsien, p. 28, ll. 5-18.

Dower

> (c) An alternative polymer stepwise synthetic strategy can be employed. In this embodiment, the fluorophores need not be removable and may be attached to irreversible chain terminators. Examples of such compounds for use in sequencing DNA include, but are not limited to, dideoxynucleotide triphosphate analogs as described by Prober et al. (1987) Science 238:336-341.

Dower, col. 25, ll. 41-47.

Under *Advanced Display*, it must be clear that Prober I is being cited in Tsien and Dower for its disclosure of deaza-substituted nucleotides. Illumina did not meet its burden in establishing this fact.

Neither of the publications cited by Illumina clearly indicates Prober I is being referenced for its teaching of deaza-substituted nucleotides. Tsien refers to Prober I for its teaching that fluorescent ddNTPs can be incorporated by two different enzymes, but did not identify the fluorescent ddNTPs as deaza-substituted as required by claims 1 and 11, or point to a passage in Prober I where the latter could be found. Dower referred to Prober I for its teaching of the use of dideoxynucleotide triphosphate analogs in DNA sequencing, but did not specifically identify the analogs as deaza-substituted nor identify where Prober I taught such analogs.

With respect to Tsien, Illumina further points to Tsien's disclosure on page 3, lines 13-16, "that the documents identified in the specification are to be used 'for their teaching of synthetic methods, coupling and detection methodologies, and the like.'" Petition 26-27. However, such generic disclosure does not remedy Tsien's failure to identify deaza-substituted analogs as the specific teaching referred for which Prober I was cited.

Illumina provided testimony by Dr. George Weinstock[13] that Prober I is incorporated by Tsien for "its teaching regarding attachment of a linker to the 7 position in the 7-deazapurine," but these words do not appear in Tsien. Weinstock Decl. ¶ 63. Dr. Weinstock did not provide a factual basis for his testimony as to why this teaching would have been recognized by one of ordinary skill in the art. *Id.*

Similarly, Dower's general statement that all documents referred to in its specification are "incorporated by reference" does not direct the ordinary skilled worker to specific disclosure in Prober I of deaza-substituted nucleotide analogs.

Because Illumina did not provide adequate evidence that Tsien and Dower clearly identified the deaza-substituted nucleotides as the material to be incorporated by their reference to Prober I, we find that there is not a reasonable likelihood that Illumina would prevail in establishing that claims 1-7, 11-12, 14-15 and 17 are anticipated by the Tsien and Dower disclosures. Since the proper standard under 35 U.S.C. § 314 was not met, we do not authorize an inter partes review to be instituted with respect to Illumina's

[13] Dr. Weinstock received a Ph.D. in Microbiology from the Massachusetts Institute of Technology in 1977. Weinstock Decl. ¶ 4, Exhibit 1021 (dated Sept. 15, 2012). At the time the declaration was executed, Dr. Weinstock was Professor of Genetics and a Professor of Molecular Microbiology and Associate Director of The Genome Institute at Washington University School of Medicine in St. Louis, Missouri. *Id.* at ¶ 3. Dr. Weinstock stated that he has "over 40 years of experience working in the field of genomics, including over 15 years managing DNA sequencing projects, and first-hand experience in the use of DNA sequencing platforms." *Id.* at ¶ 8. Dr. Weinstock's expertise is in DNA sequencing, which is the field of the claimed invention. We therefore find that Dr. Weinstock is qualified to testify in this proceeding.

patentability challenges based on Tsien and Dower as anticipatory publications.

B. Patentability challenge based on Stemple II (Petition 41)

Stemple II discloses a sequencing by synthesis method in which a nucleic acid template to be sequenced is attached to a solid support via the interaction with a polymerase which Illumina contends anticipates claims 1-7, 11, 12, 14, 15, and 17. Petition 41 & 43. Figure 3 of Stemple II, reproduced, illustrates the various elements of Stemple II's method:

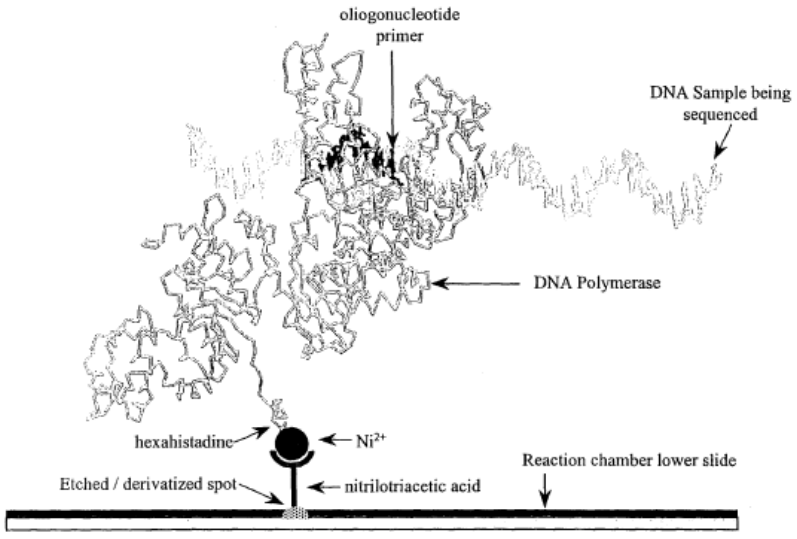

Figure 3 shows "DNA polymerase" attached to the microscope slide. Stemple II, p. 7, ll. 1-2; Petition 42. Oligonucleotide primer and DNA sample template to be sequenced are shown as "both bound to the polymerase." *Id.* at p. 7, ll. 7-9. The primer anneals to the DNA sample template and is extended by the addition of a labeled nucleotide to its 3'-OH group, catalyzed by the polymer. *Id.* at p. 11, l. 13; p. 14, l. 31 to p. 15, l. 10.

Illumina contends that the claimed requirement of a deaza-substituted nucleotide is met by Stemple II's citation to Anazawa[14] which is said to teach deaza-substituted nucleotides and to be incorporated by reference in the Stemple II document. Petition 45.

Issues

There are two principles issues in determining the sufficiency of Illumina's patentability challenge based Stemple II. The first issue is whether Stemple II describes deaza-substituted nucleotides in the manner required by 35 U.S.C. § 102(b). Since Illumina contends that deaza-substituted nucleotides are described in Stemple II because of the incorporation by reference of Anazawa, the specific question is whether Stemple II specifically identifies Anazawa for its teaching of deaza-substituted nucleotides and indicates where in the document the teaching is found. *Advanced Display*, 212 F.3d at 1282. The second issue is whether Stemple II describes "a nucleic acid template attached to a solid surface" as required by independent claims 1 and 11.

1. *Deaza-substituted nucleotides*

Stemple II, like Tsien and Dower, is not said by Illumina to disclose deaza-substituted nucleotides expressly, but rather captures such disclosure by incorporating a publication – Anazawa – which Illumina contends describes the claimed substituted nucleotides. The passage in Stemple II said by Illumina to describe deaza-substituted nucleotides is reproduced below (Petition 45):

[14] Takeshi Anazawa et al, WO 98/33939 (August 6, 1998), Exhibit 1010, citations are to English translation, Exhibit 1011.

> In an alternative configuration a photolabile group is attached to the 3'-OH using succinimide or other chemistry and a fluorochrome-photo labile linker conjugate is attached directly to the base of the nucleotide as described by Anasawa [sic, Anazawa] et al., WO 98/33939.

Stemple II, p. 31, ll. 10-12.

As with Tsien and Dower, Stemple II simply does not identify Anazawa for its teaching of deaza-substituted nucleotides.

2. *"Nucleic acid template attached to a solid surface" and "nucleic acid templates immobilized on a solid surface"*

Illumina contends that Stemple II's disclosure of nucleic acid bound to polymerase directly attached to a solid surface meets the limitations of claims 1 and 11 of nucleic acid "attached" (claim 1) or "immobilized" (claim 11) to a solid surface. Petition 43 & 47. Specifically, Illumina's position is that the disputed claim language properly is interpreted to include attachment "to a solid support via the interaction with a polymerase," as shown in Figure 3 of Stemple II (reproduced above). Petition 41.

Columbia contends that Illumina's challenged is based on an improper interpretation of the claims. Preliminary Response 9-10. Columbia contends that the claims, when read in light of the patent, require the nucleic acid to be attached to the surface, not via a polymerase molecule. *Id.*

We have construed the claimed requirement that the DNA be "attached" or "immobilized" to the solid surface to require direct attachment of the DNA *without* intervening structures. *See* Claim interpretation *supra*. There is no dispute that Stemple II describes polymerase directly attached to a solid surface, with the template DNA bound to the polymerase. The DNA therefore is fixed to the solid surface through the intervening structure of the

DNA polymerase. Since Stemple II describes attachment through intervening polymerase, Stemple II does not meet the limitations of claims 1 and 11.

Summary

Because Illumina did not provide adequate evidence 1) that Stemple II clearly identified the deaza-substituted nucleotides as the material to be incorporated by its reference to Anazawa; and 2) that Stemple II described nucleic acid template attached or immobilized to a solid support as those terms would be reasonably construed in the light of the '698 patent specification, we find that there is not a reasonable likelihood that Illumina would prevail in establishing that claims 1-7, 11-12, 14-15 and 17 are anticipated by Stemple II. Since the proper standard under 35 U.S.C. § 314 was not met, we do not authorize an inter partes review to be instituted with respect to Illumina's patentability challenges based on Stemple II as an anticipatory publication.

C. Patentability challenge based on Stemple III (Petition 50)

Stemple III issued as U.S. Patent No. 7,270,951 on September 18, 2007, as the national stage application of the PCT application that published as Stemple II. We determined that it is not reasonably likely that Illumina would prevail in establishing that a claim challenged in the petition is anticipated by Stemple II. Illumina relies on substantially the same disclosure in Stemple III as they did for Stemple II. The evidence does not show that Stemple III would be more likely than Stemple II to provide a complete description of the claimed subject matter. The rejections appear to be duplicative. Accordingly, we shall not authorize an inter parte review at

this time for the grounds based on Stemple III as an anticipatory publication. 37 C.F.R. § 42.108(a). *See* also *Liberty Mutual Ins. Co. v. Progressive Casualty Ins. Co.*, CBM-2012-00003 (Paper No. 7), at *2 (PTAB Oct. 25, 2012) (not proceeding on redundant grounds in absence of meaningful distinction); *see also* 37 C.F.R. § 42.1(b) ("This part [i.e., Part 42 of Title 37, Code of Federal Regulations] shall be construed to secure the just, speedy, and inexpensive resolution of every proceeding.")

2. OBVIOUSNESS

Illumina cites the Tsien, Dower, Stemple II, and Stemple III publications for teaching all the steps of the claimed method and immobilized nucleic acid templates, as recited in claims 1 and 11, respectively. Contingent upon the Board deciding that Illumina did not meet its burden in establishing the latter publications described deaza-substituted nucleotides as recited the claims, Illumina proposed additional grounds of rejection under 35 U.S.C. § 103. These rejections combined each of Tsien, Dower, Stemple II, and Stemple III with either of Prober I, Prober II,[15] Seela I, Hobbs,[16] or Anazawa, the latter of which Illumina contends establishes that deaza-substituted nucleotide analogues were known in the art prior to the invention. Illumina contends there was reason to have used the deaza-substituted nucleotide analogues in the sequencing methods of each of Tsien, Dower, Stemple II, and Stemple III.

The patentability challenges to the claims under 35 U.S.C. § 103 each involve the same issue of whether it would have been obvious to have

[15] James Prober et al., U.S. 5,242,796 (September 7, 1993), Exhibit 1004.
[16] Frank Hobbs et al., U.S. 5,047,519 (September 10, 1991), Exhibit 1013.

utilized deaza-substituted nucleotides in the DNA sequencing by synthesis methods of Tsien, Dower, and Stemple II, and Stemple III.

To establish obviousness under 35 U.S.C. § 103, the following factors must be taken into consideration: (a) the scope and content of the prior art; (b) the differences between the prior art and the claimed subject matter; (c) the level of skill in the pertinent art; and (d) evidence of secondary considerations. *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966). In making an obviousness determination, "it can be important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does." *KSR Int'l Co. v. Teleflex Inc*., 550 U.S. 398, 418 (2007).

We begin by summarizing Illumina's evidence of the scope and content of the prior art.

Scope and content of the prior art

A. DNA sequencing by synthesis

As discussed above, the invention of the '698 patent involves sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA by a polymerase reaction. The method is referred to as "DNA sequencing by synthesis."

To establish that DNA sequencing by synthesis was known prior to the filing date of the '698 Patent, Illumina cited the disclosure in the Background section of the '698 Patent stating that the "concept of sequencing DNA by synthesis without using electrophoresis was first revealed in 1988 (Hyman, 1988)." '698 patent, col. 2, ll. 7-9; Petition 12.

Tsien, Dower, and Stemple II were further identified by Illumina as teaching the specifically claimed DNA sequencing by synthesis methods. Petition 18, 30, 41, and 50; Tsien, Abstract & pp. 10-13; Dower, Figure 8, col. 4, ll. 44-60, col. 14, ll. 38-59, & col. 23-28; Stemple II, Abstract, p. 5, pp. 32-33, & p. 35; see also section B. infra.

Illumina also provided testimony by Dr. George Weinstock that DNA sequencing by synthesis was known prior to the earliest filing date. Dr. Weinstock testified that DNA sequencing by synthesis was known prior to the earliest filing date, and cites Dower, Tsien, and Stemple I,[17] II and III, as evidence. Weinstock Decl. ¶¶ 60, 62, 69, 72, and 75.

B. Deaza-substituted nucleotides

All the claims require a deaza-substituted nucleotide. Illumina contends that the "use of nucleotide analogues including deazapurines was well known in the nucleic acid sequencing field at least as early as the mid-1980s as shown in a wide variety of prior art documents." Petition 14. To establish this fact, Illumina cites published scientific journal articles and expert testimony. The evidence cited by Illumina as follows:

(1) *Seela I*

Seela I states that 7-deaza-2'deoxyguanosine can advantageously be used in place of regular bases in polymerase-based sequencing methods. See, e.g., Seela I at col. 2, lines 23-29 & 33-50. Seela I states that "7-deaza-2'-deoxyguanosine triphosphate can be used instead of 2'-deoxyguanosine

[17] Derek Stemple et al., U.S. Application 09/226,187 (filed March 10, 1999), Exhibit 1009.

triphosphate in those sequencing methods for DNA in which the use of DNA polymerase is necessary." *Id.* at col. 4, lines 4-7. Additionally, Seela I teaches that the 2'-deoxyguanosine triphosphate can here be replaced by 7-deaza-2'-deoxyguanosine triphosphate while using the "usual" reaction conditions. *Id.* at col. 4, lines 11-13. Seela I also states that deazaguanine-based nucleotides allow for effective sequencing of cytosine-guanine rich areas. *Id.* at col. 4, lines 31-33.

(2) *Prober I*

Prober I describes DNA sequencing methods using fluorescent tagged chain terminating reagents. Prober I, Abstract. Prober I teaches using ddNTP's to which a fluorescent label is attached to the 7-position in a 7-deazapurine. *Id*. at p. 337, col. 1. The modified labeled nucleotides were successfully incorporated into synthesized DNA by T7 DNA polymerase. *Id.* at p. 337, col. 2; p. 340, col. 1.

Prober I teaches at p. 341, column 1:

> In practice there are regions of DNA which are difficult to sequence due to aberrations in electrophoretic mobility caused by secondary structure (17). . . 2'-Deoxy-7-deazaguanosine triphosphate has been used (c^7dGTP) in place of dGTP to minimize these effects.

(3) *Prober II*

Prober II describes a process for DNA sequencing using a modification of a Sanger chain termination method, where the terminator can be a 7-deazaadenine or 7-deazaguanine which a carries a fluorescent marker attached to the 7-position of the deazapurine. Prober II, col. 7, l. 50 to col. 8, l. 39. Prober II teaches that "art shows that . . . the 7-position on

purines may carry even a relatively bulky substituent without significantly interfering with overall binding or recognition [R.M. K. Dale et al., *Proc. Nat. Acad. Sci. USA*, 70, 2238-2242 (1973)]. Prober II, col. 18, l. 64 to col. 19, l. 2.

(4) *Hobbs*

Hobbes describes using deaza-substituted nucleotides (Hobbs, col. 10, line 67 to col. 11, line 11) as chain terminating substrates for DNA sequencing using polymerase (*id.* at Abstract). Hobbs teaches attachment of a linker to the 7-position of purine nucleotides to "provide labeled chain-terminating substrates that do not interfere excessively with the degree or fidelity of substrate incorporation." *Id.* at col. 8, ll. 58-61. Hobbs discloses that the nucleotides include a heterocyclic base and that "[p]referred heterocyclic bases include: . . . 7-deazaadenine [and] (j), 7-deazaguanine . . . The unnatural 7-deazapurines can be employed to attach the linker without adding a net charge to the base portion and thereby destabilizing the glycosidic linkage." *Id.* at col. 10, line 67 - col. 11, line 4.

(5) *Anazawa*

Figure 7 of Anazawa shows a nucleotide 7-deazaguanine (natural nitrogen at position 7 replaced with a carbon) labeled with the fluorescent marker Texas Red at the 7-position. Anazawa (translation), Fig. 7 and p. 6, lines 5-6. Anazawa teaches that the labeled nucleotide "can be incorporated by polymerase-based complementary-chain elongation reactions, as has been confirmed by various experiments." *Id.* at p. 6, ll. 10-12.

(6) *Weinstock Declaration*

Dr. Weinstock stated:

> 38. The use of nucleotide analogs including deazapurine, and a label attached at the 7-position thereof, was known in the nucleic acid sequencing field at least as early as the late 1980s.
>
> 43. It was widely known to use deazapurine-based nucleotides in the sequencing by synthesis methods at least 10 years prior to the filing date of the '698 patent. See e.g., Dower, Tsien, Stemple II and the references cited therein, including Prober I and Anazawa.
>
> 44. Multiple prior art references recognized a number of advantages for using deazapurines as the base in nucleotide analogs for DNA sequencing.

To support paragraph 44, Dr. Weinstock cited specific passages in Seela I, Prober I, Hobbs, and Prober II, which show that deazapurines were used in DNA sequencing and were capable of being incorporated into DNA by polymerase during the sequencing reaction. Weinstock Decl., ¶¶ 45-49 & 52-55.

C. Summary

We credit Dr. Weinstock's testimony and find persuasive the other evidence discussed above which sufficiently establish that 1) DNA sequencing by synthesis was known in the art; and 2) that deaza-substituted nucleotides had been used in DNA sequencing prior to the earliest filing date of the '698 Patent. We note that in the Preliminary Response under 37 C.F.R. § 42.107 Columbia did not dispute Illumina's evidence of 1) and 2) as summarized above. In sum, we find that 1) and 2) are representative of the scope and content of the prior art at the time the '698 Patent was filed.

Reason to combine & analysis

It is important to have a reason to have combined the cited publications to have arrived at the claimed invention. *KSR*, 550 U.S. at 418. Illumina's reasons for combining the prior art, and the sufficiency of these reasons, are addressed below.

D. Patentability challenge based on Tsien and Prober I (Petition 27)

The issue is whether it would have been obvious to one of ordinary skill in the art to have used Prober I's deaza-modified nucleotides in Tsien's sequencing method. Illumina's evidence that the sequencing method of claim 1 and the sequencing product of claim 11 of the '698 patent are obvious in view of Tsien and Prober I includes:

● Evidence that DNA sequencing by synthesis methods were taught in the prior art before the earliest filing date of the '698 Patent, including substantial evidence that all the steps of the claimed method were described by Tsien. *Supra* section A; Petition 18-22. We note that this evidence is not disputed by Columbia.

● Evidence that deaza-substituted nucleotides had been used in DNA sequencing prior to the earliest filing date of the '698 Patent. *Supra* section B; Petition 14-17. We note that this evidence is not disputed by Columbia.

● Specific disclosure by Prober I of a fluorescent label attached to the 7-position of deaza purines, a deaza-substituted nucleotide. *Supra* section B(2); Petition 14-17.

● Disclosure by Prober I that fluorescent ddNTPs could be incorporated by polymerase into DNA. *Supra* section B(2); Petition 28-29.

Illumina also provided evidence of a reason to have utilized a deaza-substituted nucleotide in Tsien's sequencing method:

> Tsien states that Prober I is used "for [its] teaching of synthetic methods, coupling and detection methodologies, and the like." Tsien, p. 3, ll. 11-16. With specific reference to Prober I, at page 28, lines 5-18. Tsien states that:
>
> > One method involves the use of a fluorescent tag attached to the base moiety…. This method is included because a number of base moiety derivatized dNTP analogues have been reported to exhibit enzymatic competence. Prober et al. (1987) [Prober I] show enzymatic incorporation of fluorescent ddNTPs by … Sequenase™
>
> Tsien thus provides an express teaching, suggestion, and motivation to combine Tsien with the disclosures of Prober I with respect to "base moiety derivatized" nucleotide analogues. See Tsien at page 3, ll. 14-16 and page 28, ll. 16-18, respectively; see Weinstock Decl. ¶¶ 65-67.

Petition 28.

Illumina has established that deaza-substituted were effective substrates for DNA polymerase in DNA sequencing. See Section B *supra*. Illumina also has established that Tsien reasonably suggested using Prober I's dNTP analogues, which included labeled deazapurines, in its method. Tsien, p. 28, lines 16-18; p. 29, lines 10-14. Thus, Illumina's reasoning, coupled with content of the prior art and the specific teachings of Prober I, are sufficient to establish a reasonable likelihood, the pertinent standard under 35 U.S.C. § 314, that they will prevail in showing that claims 1 and 11 are unpatentable under 35 U.S.C. § 103. Accordingly, we authorize an inter partes review of claims 1 and 11 to be instituted with respect to Illumina's patentability challenge based on Tsien and Prober I.

With respect to dependent claim 2-7, 12, 14, 15, and 17, Illumina identified specific disclosure in Tsien where each limitation is found. Petition 22-26. We find Illumina's factual assertions to be supported, and authorize an inter partes review of claims 2-7, 12, 14, 15, and 17 to be instituted with respect to Illumina's patentability challenge based on Tsien and Prober I.

E. Patentability challenge based on Tsien and Prober II (Petition 54)

The issue is whether it would have been obvious to one of ordinary skill in the art to have used Prober II's deaza-modified nucleotides in Tsien's sequencing method. Illumina states that the reason to combine Tsien and Prober II are the same as for the combination of Tsien and Prober I. Petition 53 & 54. The evidence does not show that Tsien in combination with Prober II would be more likely than the Tsien in combination with Prober I to establish the obviousness of the claimed subject matter. The rejections appear to be duplicative. Accordingly, as the reason for combining the publications are the same, and because Prober I and Prober II were each relied upon for their teachings of deaza-substituted nucleotides, we shall not authorize an inter partes review at this time for the grounds based on the combination of Tsien and Prober II. *See* 37 C.F.R. § 42.108(a) and discussion above about duplicative rejections.

F. Patentability challenge based on Tsien and Seela I (Petition 56)

The issue is whether it would have been obvious to one of ordinary skill in the art to have used Seela I's deaza-modified nucleotides in Tsien's sequencing method. Illumina's evidence that the sequencing method of

claim 1 and the sequencing product of claim 11 of the '698 patent were obvious at the time of the invention includes:

● Evidence that DNA sequencing by synthesis methods were taught in the prior art before the earliest filing date of the '698 Patent, including substantial evidence that all the steps of the claimed method were described by Tsien. *Supra* section A; Petition 18-22. We note that this evidence is not disputed by Columbia.

● Evidence that deaza-substituted nucleotides had been used in DNA sequencing prior to the earliest filing date of the '698 Patent. *Supra* section B; Petition 14-17. We note that this evidence is not disputed by Columbia.

● Specific disclosure by Seela I that 7-deaza-2'deoxyguanosine can be used in place of regular bases in polymerase-based sequencing methods and that deazaguanine-based nucleotides allow for effective sequencing of cytosine-guanine rich areas. *Supra* section B(1); Petition 55-56.

Illumina states that the reason for combining Tsien and Seela I are the same as those for Dower and Seela. Petition 56. That reason is as follows:

> Seela I is directed toward nucleotide analogues to be used for sequencing methods. Seela I discloses use of a deazapurine for sequencing . . . [citing Seela I abstract & col. 4, lines 4-7]. Seela I expressly teaches an advantage of using the 7-deaza-2' deoxyguanosine stating "by using the compounds according to the present invention, a disturbance-free sequencing of cytosine-guanine-rich nucleic acids is possible." Seela I, col. 4, ll. 31-33. Thus, it would have been obvious for one of ordinary skill in the art to use the deaza nucleotides of Seela I with the polymerase medicated [sic, mediated] DNA sequencing method of Dower as expressly stated in Seela I. In addition, [i]t would have been obvious to combine Dower and Seela I [for] all the reasons discussed in section IV.2. (Tsien and Prober I) above.

Petition 55-56.

Illumina established that deaza-substituted were effective substrates for DNA polymerase in DNA sequencing. See Section B *supra*. Illumina also established that the skilled worker would have had reason to use Seela I's deaza-substituted nucleotides in Tsien's sequencing method for their advantage in sequencing cytosine-guanine regions. Thus, Illumina's reasoning, coupled with content of the prior art and the specific teachings of Seela I, are sufficient to establish a reasonable likelihood, the pertinent standard under 35 U.S.C. § 314, that they will prevail in showing that claims 1 and 11 are unpatentable under 35 U.S.C. § 103. Accordingly, we authorize an inter partes review of claims 1 and 11 to be instituted with respect to Illumina's patentability challenge based on Tsien and Seela I.

With respect to claims dependent claim 2-7, 12, 14, 15, and 17, Illumina identified specific disclosure in Tsien where each limitation is found. Petition 22-26. We find Illumina's assertions to be factually supported, and authorize an inter partes review of claim 2-7, 12, 14, 15, and 17 to be instituted with respect to Illumina's patentability challenge based on Tsien and Seela I.

G. Patentability challenge based on Tsien and Hobbs (Petition 58)

The issue is whether it would have been obvious to one of ordinary skill in the art to have used Hobbs's deaza-modified nucleotides in Tsien's sequencing method. Hobbs teaches:

> . . . various advantages of attaching the label group via a linker to the 7-deazapurine. For example, Hobbs teaches that "The unnatural 7-deazapurines can be employed to attach the linker without adding a net charge to the base portion and thereby destabilizing the glycosidic linkage." See Hobbs, col. 11, ll. 1-4 (emphasis added). Further, Hobbs teaches that "the 7-position

> of the purine nucleotides provide labeled chain-terminating substrates that do not interfere excessively with the degree or fidelity of substrate incorporation." Hobbs, col. 8, ll. 57-60.

Petition 58.

Illumina states that the reason for combining Tsien and Hobbs are the same as those for Dower and Hobbs. Petition 58-59. The reason was stated as follows:

> [I] it would have been obvious to combine Dower and Hobbs for all the reasons discussed in section IV.2. (Tsien and Prober I) above.

Petition 58. In other words, the reason for combining Tsien and Hobbs appears to be substantially the same reason as for Tsien and Prober I.

The evidence does not show that Tsien in combination with Hobbs would be more likely than the Tsien in combination with Prober I to establish the obviousness of the claimed subject matter. The rejections appear to be duplicative. Accordingly, as the reason for combining the publications are the same, and Prober I and Hobbs were each relied upon for their teachings of deaza-substituted nucleotides, we shall not authorize an inter partes review at this time for the grounds based on the combination of Tsien and Hobbs. *See* 37 C.F.R. § 42.108(a) and discussion above about duplicative rejections.

H. Patentability challenge based on Tsien, Prober I, and Rabani (Petition 52)

Illumina challenges the patentability of claims 5 and 12 based on Tsien, Prober I, and Rabani. Petition 52. Claims 5 and 12 read as follows:

> 5. The method of claim 1, wherein in step a) a plurality of different nucleic acid templates are attached to the solid surface.
>
> 12. The plurality of nucleic acids of claim 11, wherein said plurality are present in a microarray.
>
> For the specific limitations in claims 5 and 12, Illumina cited Rabani.

Illumina stated:

> Rabani identifies that "[t]he invention relates to the massively parallel single molecule examination of associations or reactions between large numbers of first complex molecules, which may be diverse." Rabani, page 1, ll. 4-6 (emphasis added). Rabani also discloses detection of "multiple probes (i.e. in arrays …)." Rabani, p. 11, ll. 3-15 (emphasis added). Modifying the sequencing process taught by Tsien to either sequence a large number of diverse molecules or to use an array format as taught by Rabani would be obvious because it is merely the use of known techniques to improve the similar Tsien systems and methods in the same way that the known features improve the methods and reagents of Rabani. See Weinstock Decl. ¶ 77.

Petition 52.

Illumina's position is fact-based and supported by the evidence. Columbia does not challenge Illumina's statement of the rejection or the underlying facts. As we discern no flaw, we conclude that the information presented by Illumina in their petition under 35 U.S.C. § 312 establishes a reasonable likelihood, the pertinent standard under 35 U.S.C. § 314, that they will prevail in showing that claims 5 and 12 are unpatentable under 35 U.S.C. § 103 in view of Tsien, Prober I, and Rabani. Accordingly, we authorize an inter partes review of claims 5 and 12 to be instituted with respect to Illumina's patentability challenges based on Tsien l, Prober I, and Rabani.

I. Patentability challenges based on Dower, Stemple II, and Stemple III

Dower, Stemple II, and Stemple III, like Tsien, are each said to describe DNA sequencing by synthesis. Additional publications are cited by Illumina for teaching deaza-substituted nucleotides, which evidence cited by Illumina establishes were known to be useful in sequencing reactions prior to the earliest filing date of the '698 patent. All three publications – Dower, Stemple II, and Stemple III – are being relied upon by Illumina for substantially the same set of facts and appear to be largely duplicative in the facts for which they are cited. Each were also published more than a year before the filing date of the '698 Patent. The evidence does not show that one publication would be more likely than the other to establish the obviousness of the claimed subject matter. Accordingly, for this reason and because the rejections appear to be largely duplicative, we shall not authorize an inter partes review at this time for the grounds based on the combinations of:

Dower with either of Prober I, Prober II, Seela I, and Hobbs;

Stemple II with either of Anazawa, Prober I, Prober II, Seela I, and Hobbs;

Stemple III with either of Prober I, Prober II, Seela I, and Hobbs.

See 37 C.F.R. § 42.108(a) and discussion above about duplicative rejections.

SUMMARY

I. Inter partes review is authorized for claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien and Prober I.

II. Inter partes review is authorized for claims 5 and 12 under 35 U.S.C. § 103(a) as obvious in view of Tsien, Prober I, and Rabani.

III. Inter partes review is authorized for claims 1-7, 11, 12, 14, 15, and 17 under 35 U.S.C. § 103(a) as obvious in view of Tsien and Seela I.

Inter partes review is not authorized for any patentability challenge or ground not specified in I, II, and III.

It is ORDERED that an initial conference call with the Board is scheduled for April 16, 2013 at 2:00 pm EST; the parties are directed to the Office Trial Practice Guide, 77 Fed. Reg. 48756, 48765-66 (Aug. 14, 2012) for guidance in preparing for the initial conference call, and should come prepared to discuss any proposed changes to the Scheduling Order entered herewith and any motions the parties anticipate filing during the trial.

Case IPR2012-00006
U.S. Patent 7,713,698

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US007713698B2

(12) **United States Patent**
Ju et al.

(10) **Patent No.: US 7,713,698 B2**
(45) **Date of Patent: May 11, 2010**

(54) **MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA**

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(*) Notice: Subject to any disclaimer, the term of this patent is extended or adjusted under 35 U.S.C. 154(b) by 0 days.

(21) Appl. No.: **11/894,690**

(22) Filed: **Aug. 20, 2007**

(65) **Prior Publication Data**

US 2008/0131895 A1 Jun. 5, 2008

Related U.S. Application Data

(60) Continuation of application No. 10/702,203, filed on Nov. 4, 2003, now Pat. No. 7,345,159, which is a division of application No. 09/972,364, filed on Oct. 5, 2001, now Pat. No. 6,664,079, and a continuation-in-part of application No. 09/684,670, filed on Oct. 6, 2000, now abandoned.

(60) Provisional application No. 60/300,894, filed on Jun. 26, 2001.

(51) **Int. Cl.**

C12Q 1/68	(2006.01)
C12P 19/34	(2006.01)
C07H 21/02	(2006.01)
C07H 21/04	(2006.01)
C07H 19/04	(2006.01)
C07G 3/00	(2006.01)
C07H 21/00	(2006.01)

(52) **U.S. Cl.** **435/6**; 435/91.1; 435/91.2; 536/4.1; 536/23.1; 536/24.3; 536/24.33; 536/25.3; 536/26.6

(58) **Field of Classification Search** 435/6, 435/91.1, 91.2; 536/23.1, 24.3, 24.33, 25.3, 536/4.1, 26.6
See application file for complete search history.

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(57) **ABSTRACT**

This invention provides methods for attaching a nucleic acid to a solid surface and for sequencing nucleic acid by detecting the identity of each nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction. The invention also provides nucleotide analogues which comprise unique labels attached to the nucleotide analogue through a cleavable linker, and a cleavable chemical group to cap the —OH group at the 3'-position of the deoxyribose.

17 Claims, 28 Drawing Sheets

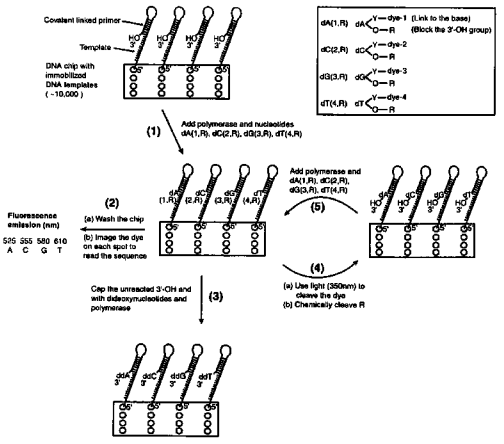

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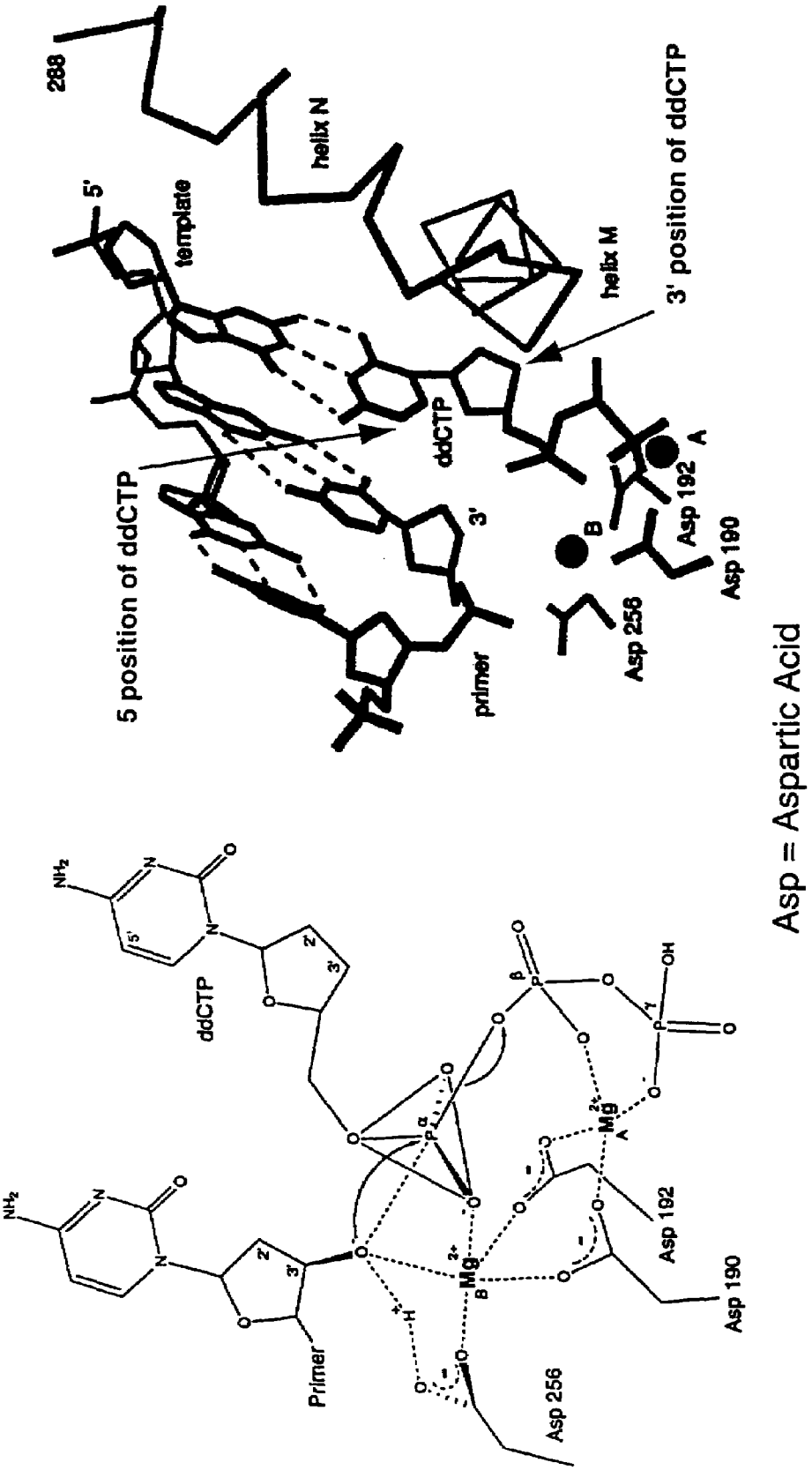
FIGURE 1


288
helix N
5'
template
helix M
3' position of ddCTP
ddCTP
5 position of ddCTP
3'
primer
Asp 192
A
B
Asp 190
Asp 256
Asp = Aspartic Acid
ddCTP
Primer
Asp 192
Asp 190
Asp 256

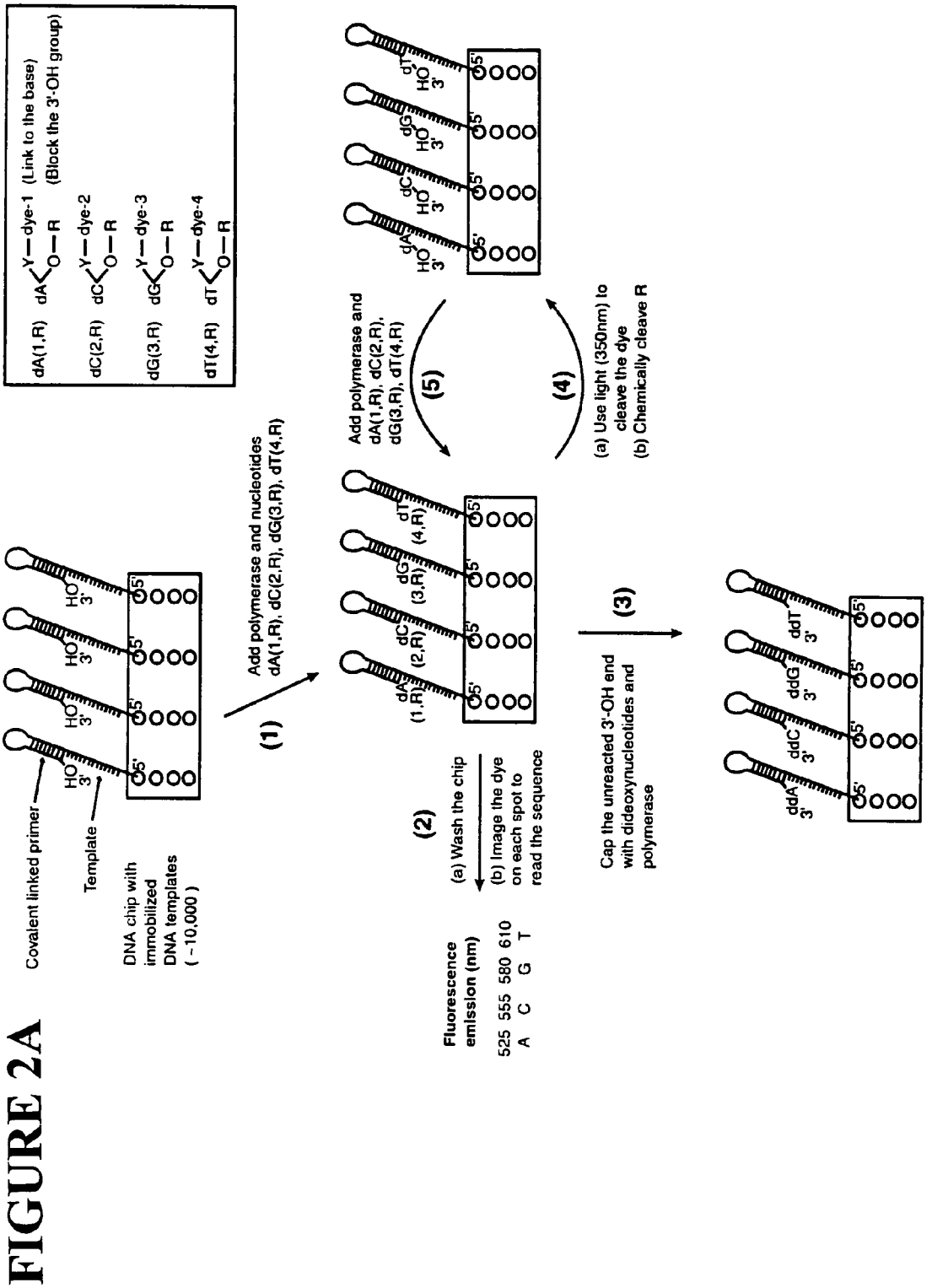

dA(1,R) dA Y—dye-1 O—R (Link to the base) (Block the 3'-OH group)
dC(2,R) dC Y—dye-2 O—R
dG(3,R) dG Y—dye-3 O—R
dT(4,R) dT Y—dye-4 O—R
Covalent linked primer
Template
DNA chip with immobilized DNA templates (~10,000)
(1)
Add polymerase and nucleotides dA(1,R), dC(2,R), dG(3,R), dT(4,R)
(2)
(a) Wash the chip
(b) Image the dye on each spot to read the sequence
Fluorescence emission (nm)
525 555 580 610
A C G T
(3)
Cap the unreacted 3'-OH end with dideoxynucleotides and polymerase
(4)
(a) Use light (350nm) to cleave the dye
(b) Chemically cleave R
(5)
Add polymerase and dA(1,R), dC(2,R), dG(3,R), dT(4,R)

FIGURE 2A

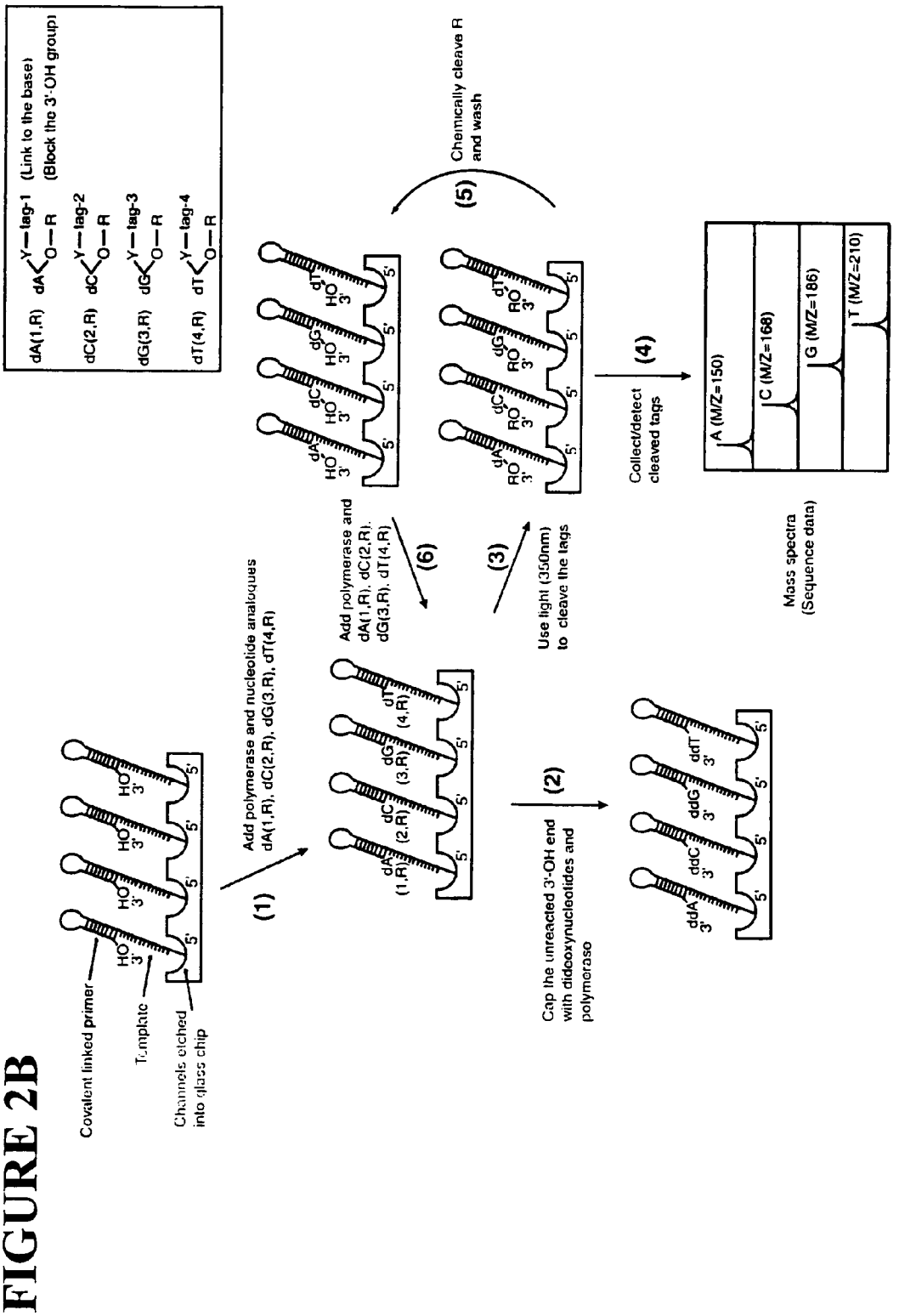

dA(1,R) dA Y—tag-1 (Link to the base)
O—R (Block the 3'-OH group)
dC(2,R) dC Y—tag-2
O—R
dG(3,R) dG Y—tag-3
O—R
dT(4,R) dT Y—tag-4
O—R
Covalent linked primer
Template
Channels etched into glass chip
(1)
Add polymerase and nucleotide analogues dA(1,R), dC(2,R), dG(3,R), dT(4,R)
(2)
Cap the unreacted 3'-OH end with dideoxynucleotides and polymerase
(3)
Use light (350nm) to cleave the tags
(4)
Collect/detect cleaved tags
A (M/Z=150)
C (M/Z=168)
G (M/Z=186)
T (M/Z=210)
Mass spectra (Sequence data)
(5)
Chemically cleave R and wash
(6)
Add polymerase and dA(1,R), dC(2,R), dG(3,R), dT(4,R)

FIGURE 2B

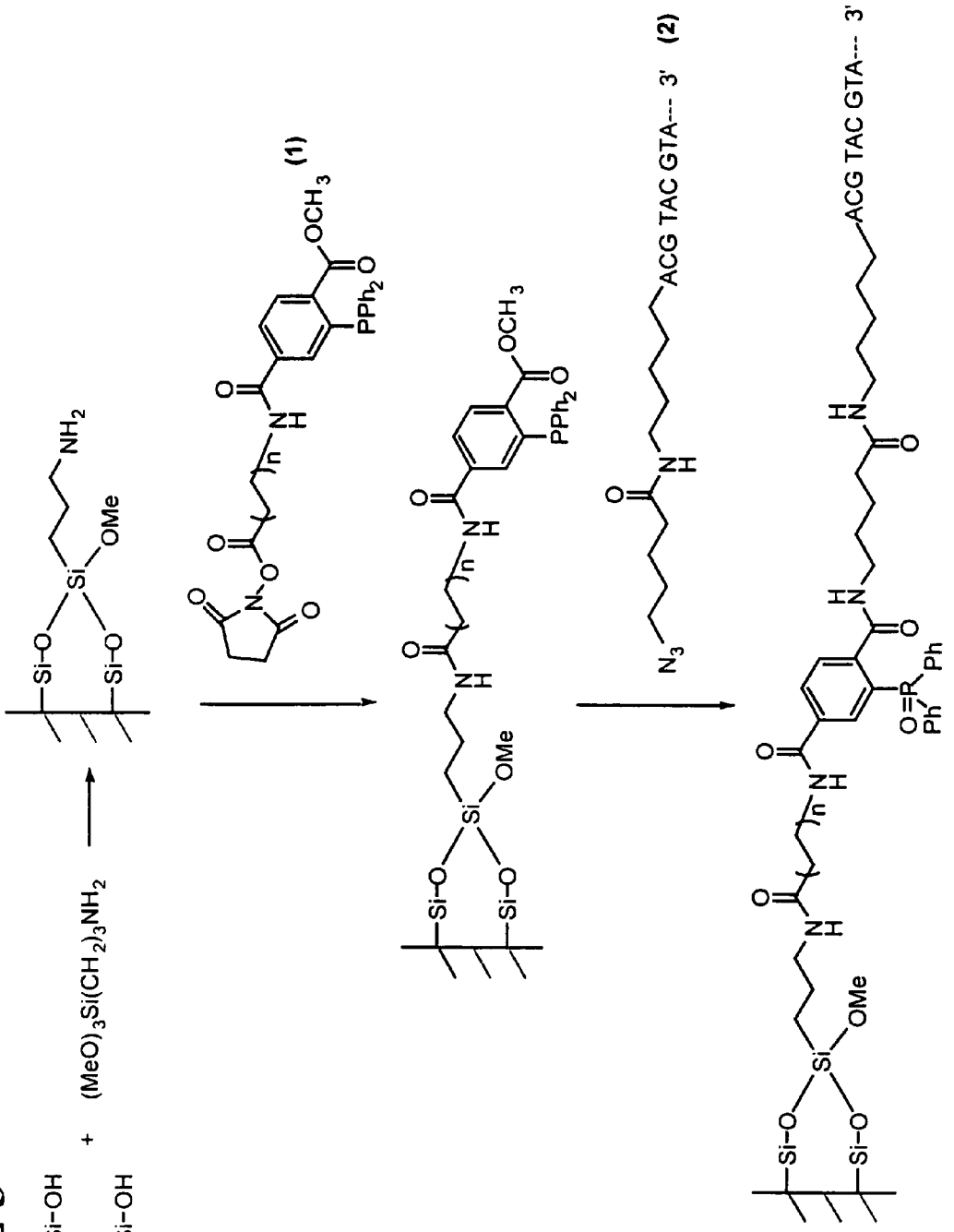

Si-OH
Si-OH
+ (MeO)3Si(CH2)3NH2
NH2
OMe
Si-O
Si-O
(1)
OCH3
PPh2
O
NH
n
ACG TAC GTA--- 3'
(2)
N3
Ph
R
O=P
Ph

FIGURE 3

FIGURE 4

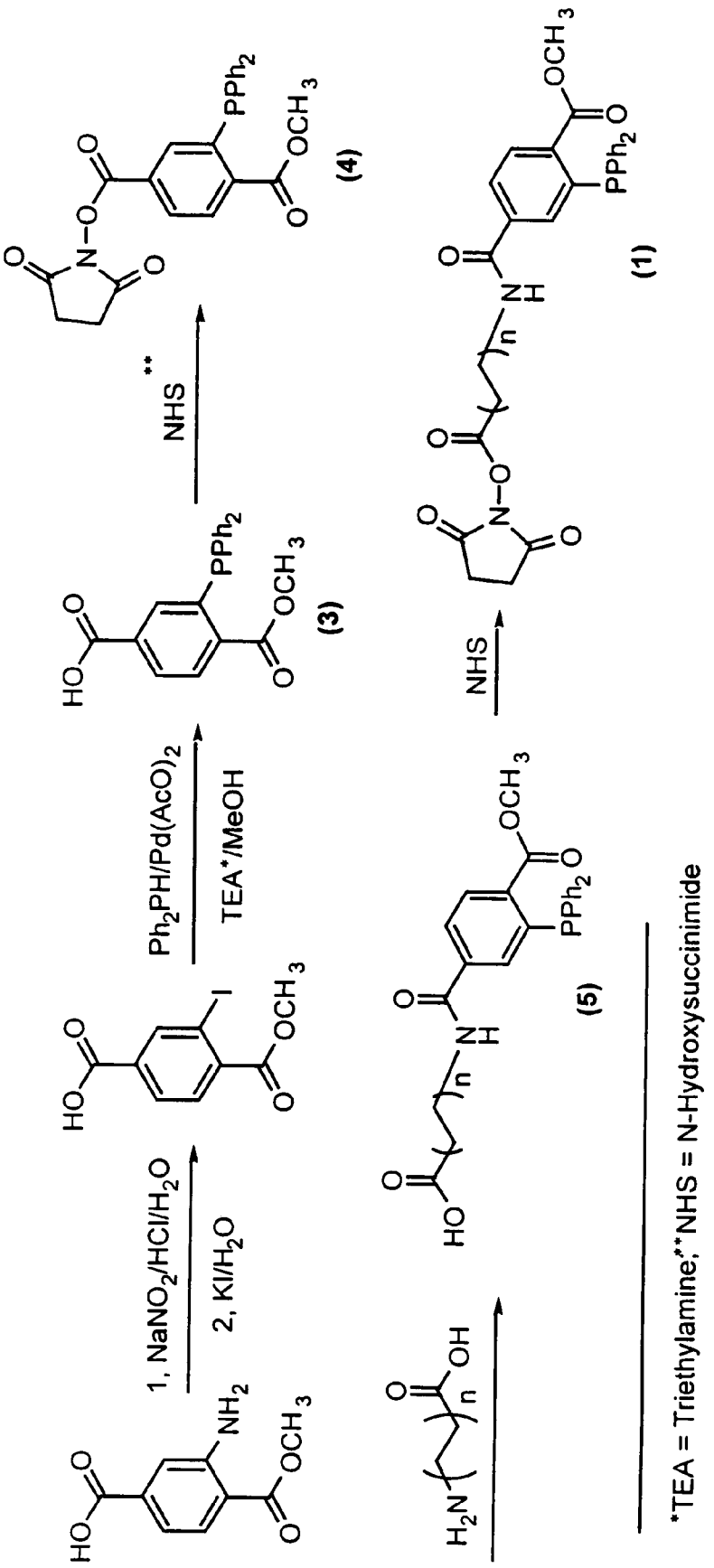
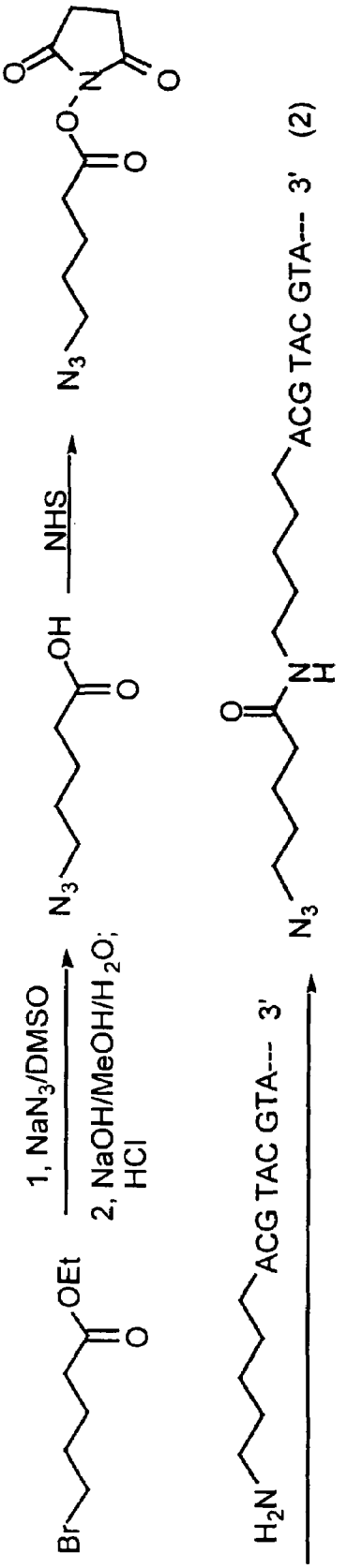

1, $NaNO_2$/HCl/H_2O
2, KI/H_2O
Ph_2PH/$Pd(AcO)_2$
TEA*/MeOH
NHS
**
NHS
(1)
(3)
(4)
(5)
*TEA = Triethylamine;**NHS = N-Hydroxysuccinimide

FIGURE 5


Br
OEt
O
1, NaN3/DMSO
2, NaOH/MeOH/H2O;
HCl
N3
OH
O
NHS
O
O
N
O
O
N3
H2N
ACG TAC GTA--- 3'
O
N
H
ACG TAC GTA--- 3' (2)
N3

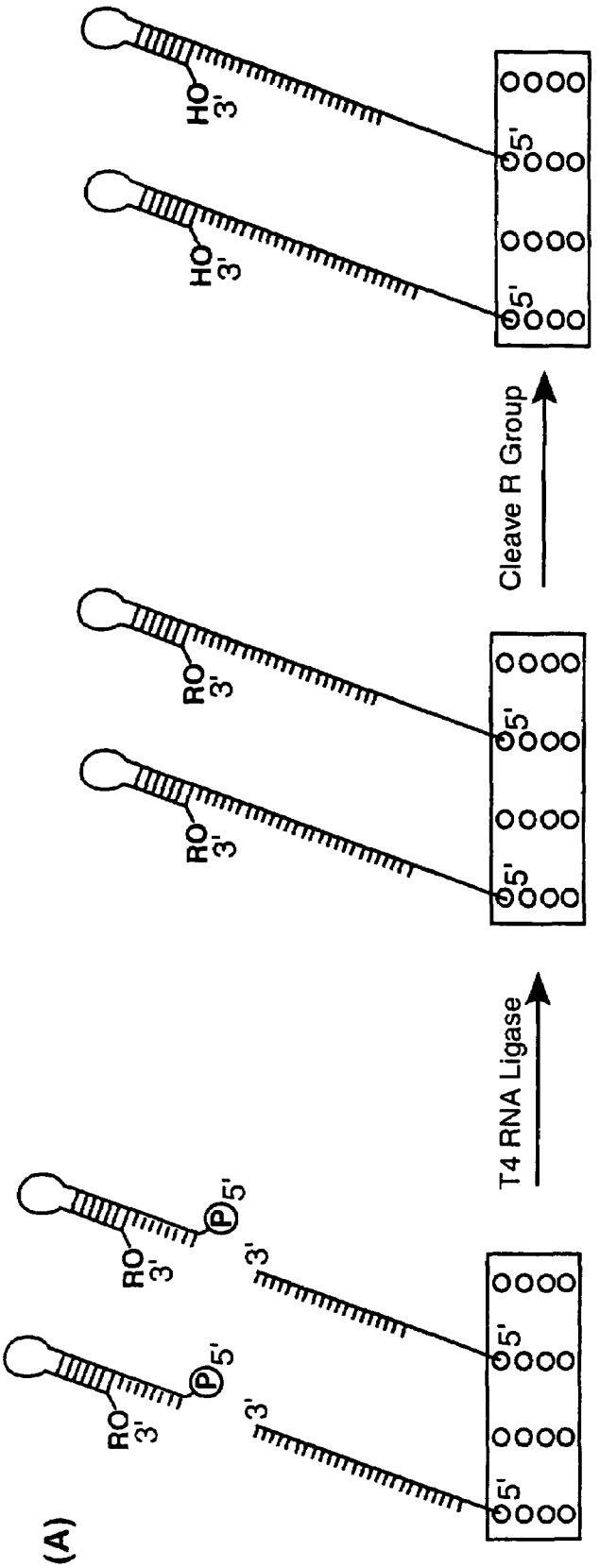


FIGURE 6A

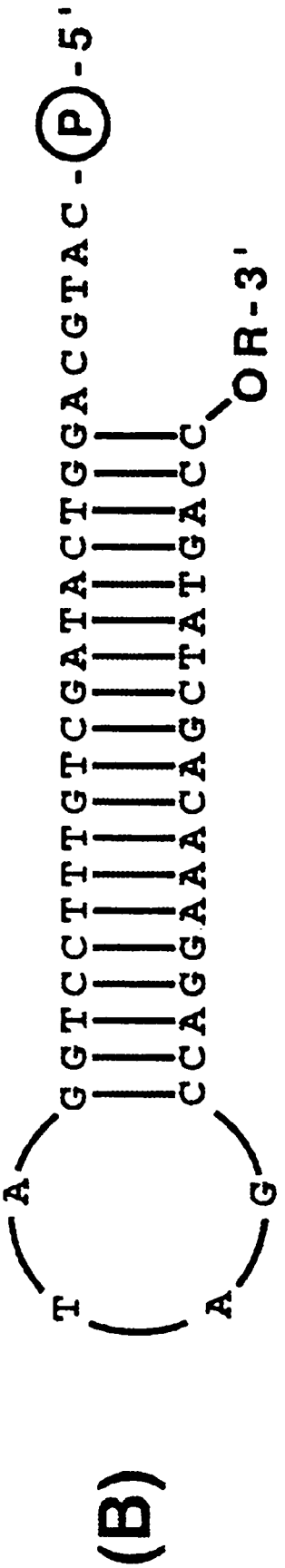
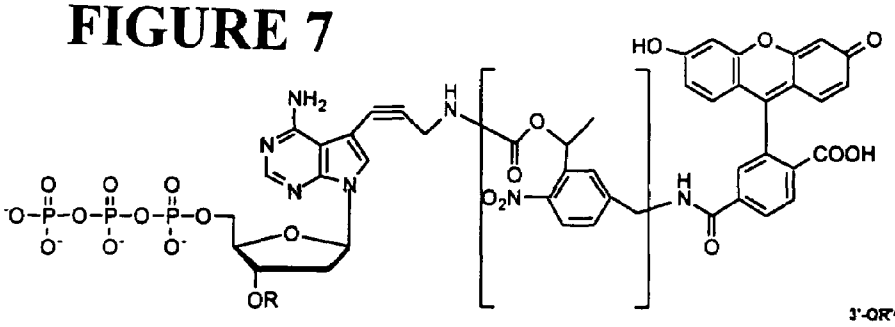

(B)
5'-(P)
GGTCCTTTGTCGATACTGGACGTAC-
CCAGGAACAGCTATGACC-OR-3'
A
G
T
A

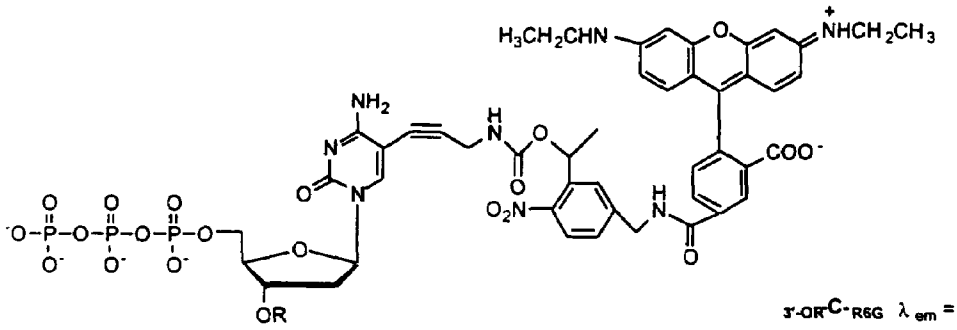
FIGURE 6B

FIGURE 7

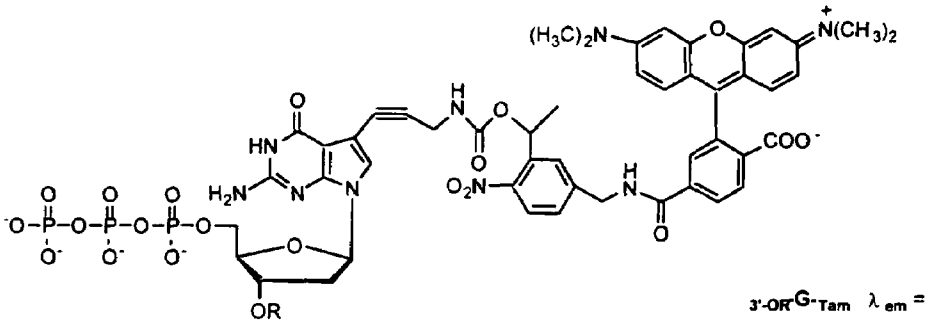


3'-OR^{A}-$_{Fam}$ λ_{em} = 525 nm

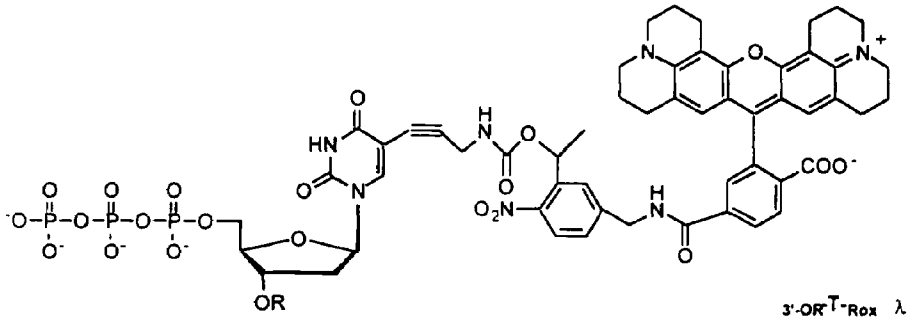
Photocleavable Linker



3'-OR^{C}-$_{R6G}$ λ_{em} = 555 nm



3'-OR^{G}-$_{Tam}$ λ_{em} = 585 nm



3'-OR^{T}-$_{Rox}$ λ_{em} = 604 nm

R = H, CH_2OCH_3 (MOM) or CH_2-CH=CH_2 (Allyl)

FIGURE 8

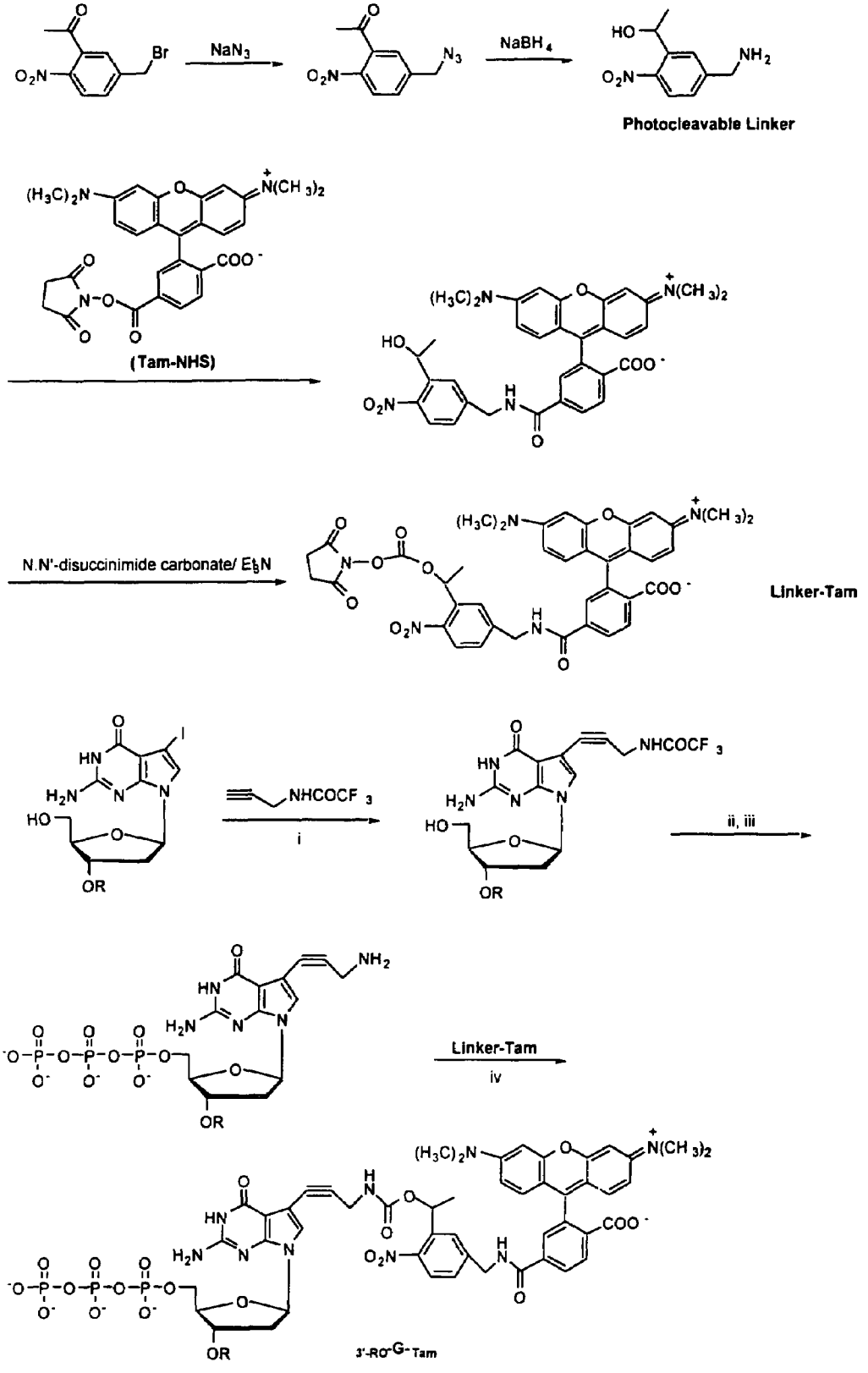
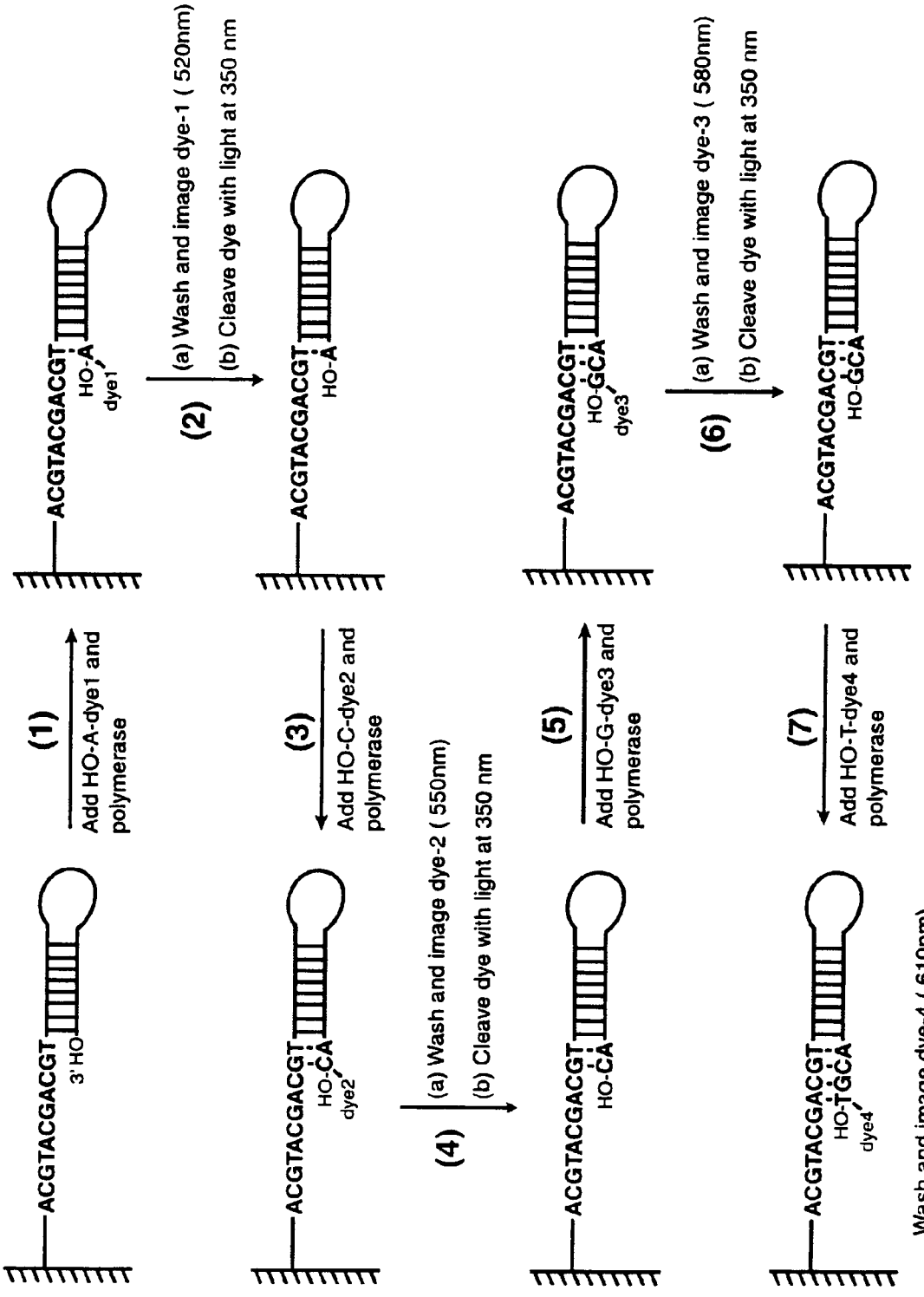

NaN3
NaBH4
Photocleavable Linker
(Tam-NHS)
N.N'-disuccinimide carbonate/ Et3N
Linker-Tam
i
ii, iii
iv
Linker-Tam
3'-RO-G-Tam

FIGURE 9


ACGTACGACGT
3' HO
(1)
Add HO-A-dye1 and polymerase
ACGTACGACGT
HO-A
dye1
(2)
(a) Wash and image dye-1 (520nm)
(b) Cleave dye with light at 350 nm
ACGTACGACGT
HO-A
(3)
Add HO-C-dye2 and polymerase
ACGTACGACGT
HO-CA
dye2
(4)
(a) Wash and image dye-2 (550nm)
(b) Cleave dye with light at 350 nm
ACGTACGACGT
HO-CA
(5)
Add HO-G-dye3 and polymerase
ACGTACGACGT
HO-GCA
dye3
(6)
(a) Wash and image dye-3 (580nm)
(b) Cleave dye with light at 350 nm
ACGTACGACGT
HO-GCA
(7)
Add HO-T-dye4 and polymerase
ACGTACGACGT
HO-TGCA
dye4
Wash and image dye-4 (610nm)

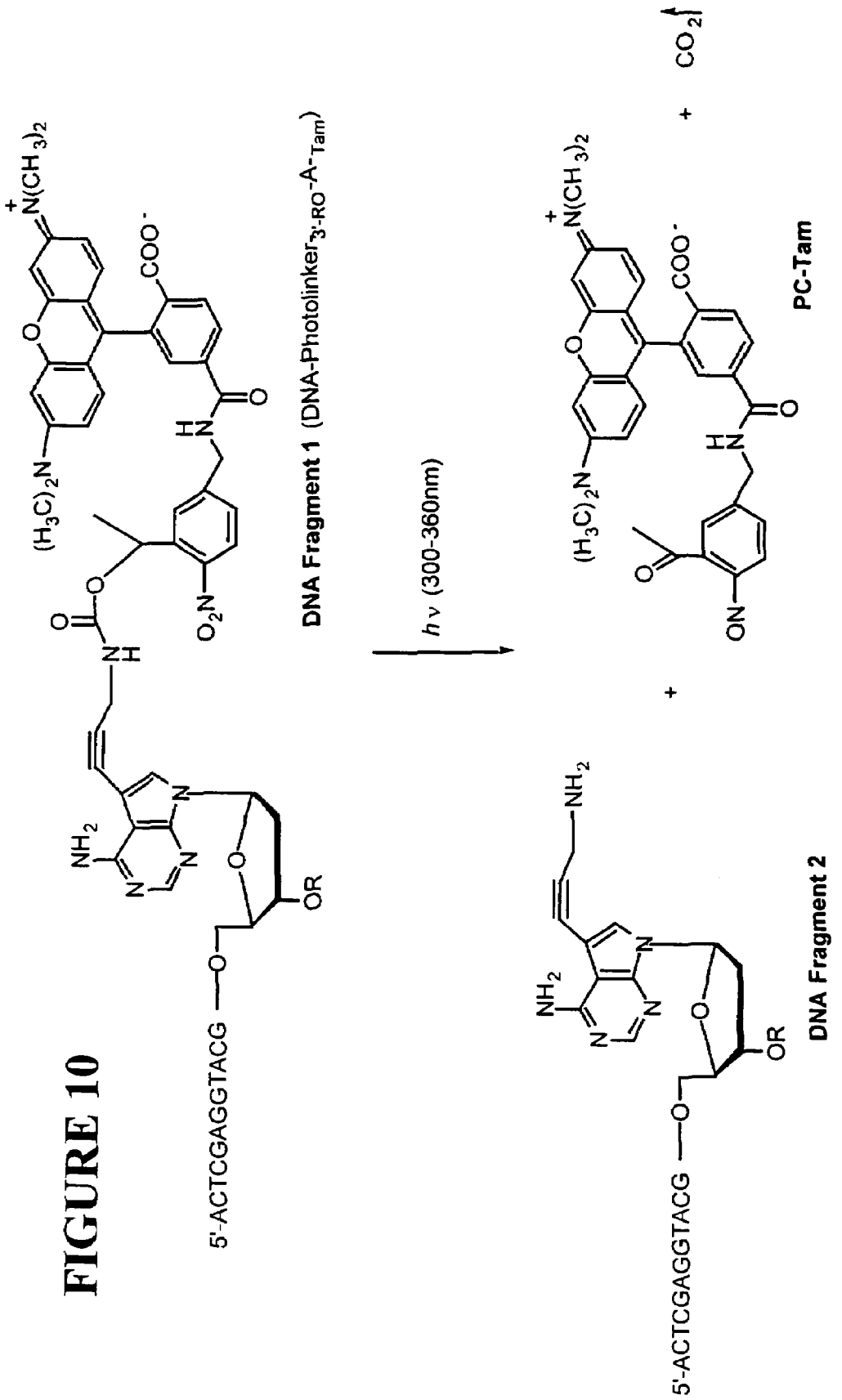

DNA Fragment 1 (DNA-Photolinker3'-RO-A-Tam)
hν (300-360nm)
PC-Tam
DNA Fragment 2
5'-ACTCGAGGTACG

FIGURE 10

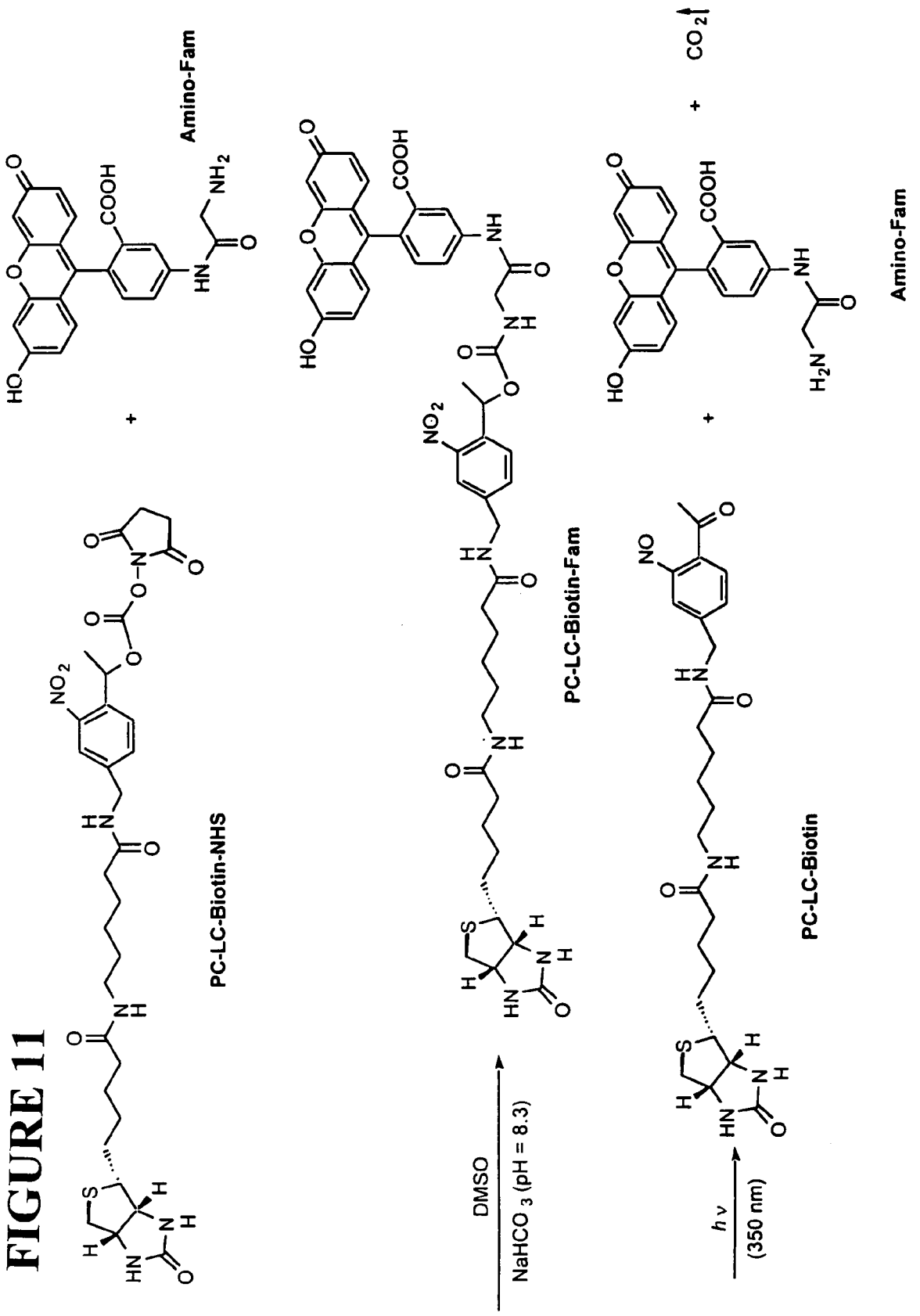

Amino-Fam
PC-LC-Biotin-NHS
DMSO
NaHCO 3 (pH = 8.3)
PC-LC-Biotin-Fam
hν
(350 nm)
PC-LC-Biotin
CO2
Amino-Fam

FIGURE 11

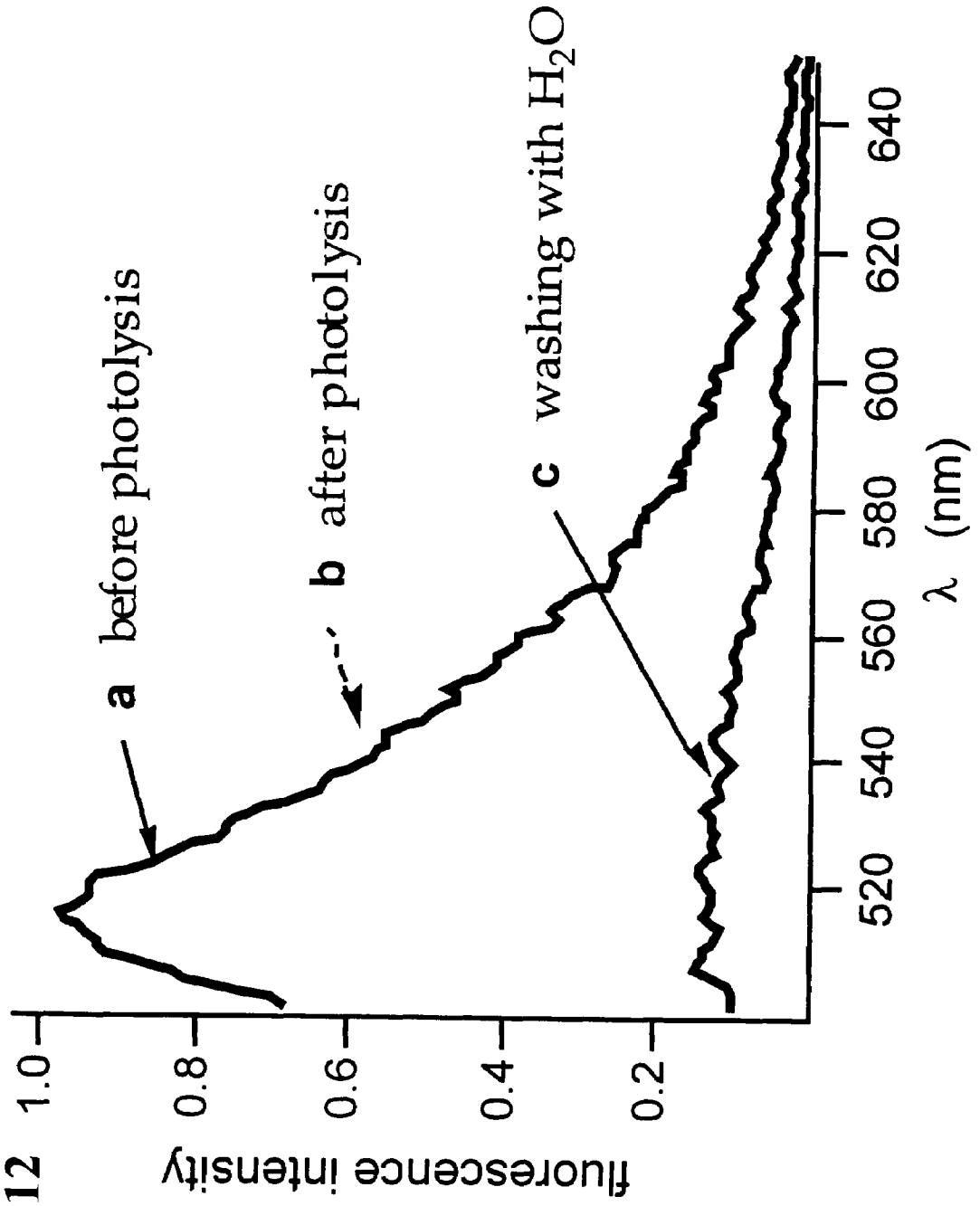
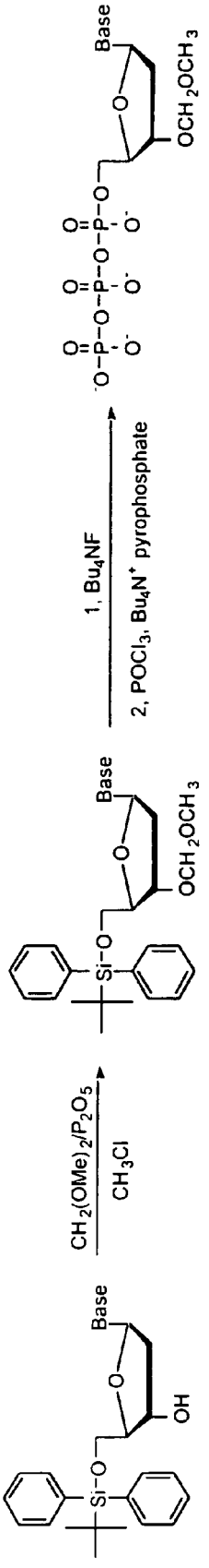

a before photolysis
b after photolysis
c washing with H_2O
fluorescence intensity
1.0
0.8
0.6
0.4
0.2
520
540
560
580
600
620
640
λ (nm)

FIGURE 12

FIGURE 13A


Base
OH
Si–O
$CH_2(OMe)_2/P_2O_5$
CH_3Cl
Base
OCH_2OCH_3
Si–O
1, Bu_4NF
2, $POCl_3$, Bu_4N^+ pyrophosphate
Base
OCH_2OCH_3

(A)

FIGURE 13B

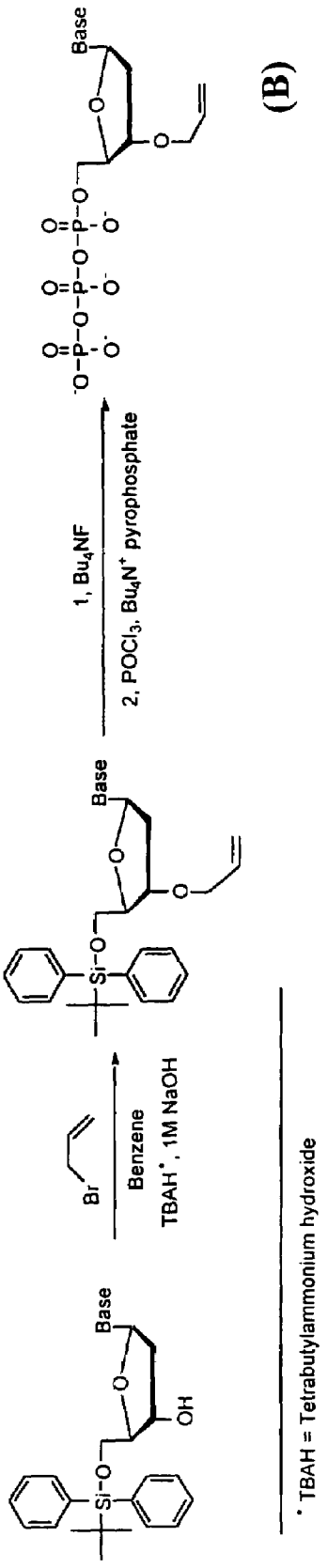
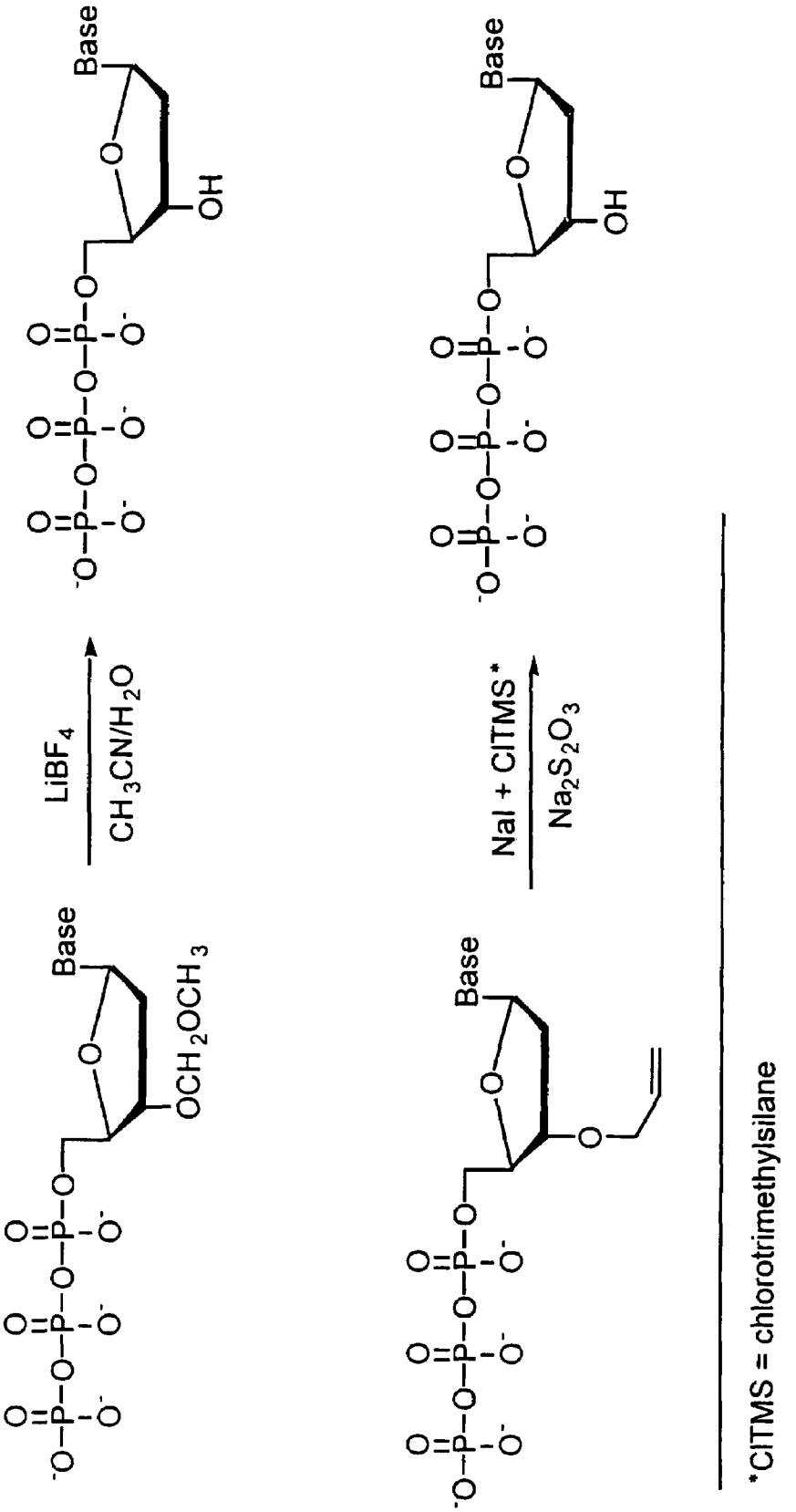

Base
OH
Si
O
Br
Benzene
TBAH*, 1M NaOH
1, Bu4NF
2, POCl3, Bu4N+ pyrophosphate
(B)
* TBAH = Tetrabutylammonium hydroxide

FIGURE 14


Base
OH
LiBF4
CH3CN/H2O
Base
OCH2OCH3
NaI + ClTMS*
Na2S2O3
Base
O
*ClTMS = chlorotrimethylsilane

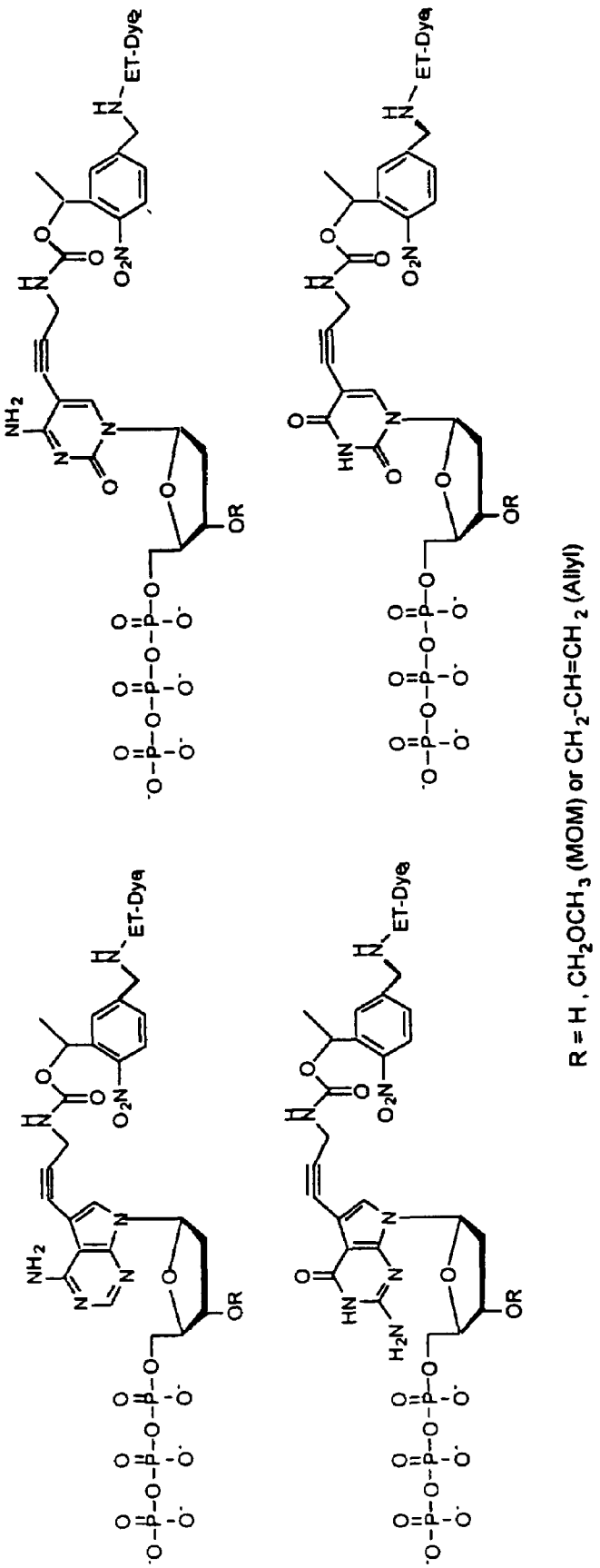


FIGURE 15A

FIGURE 15B

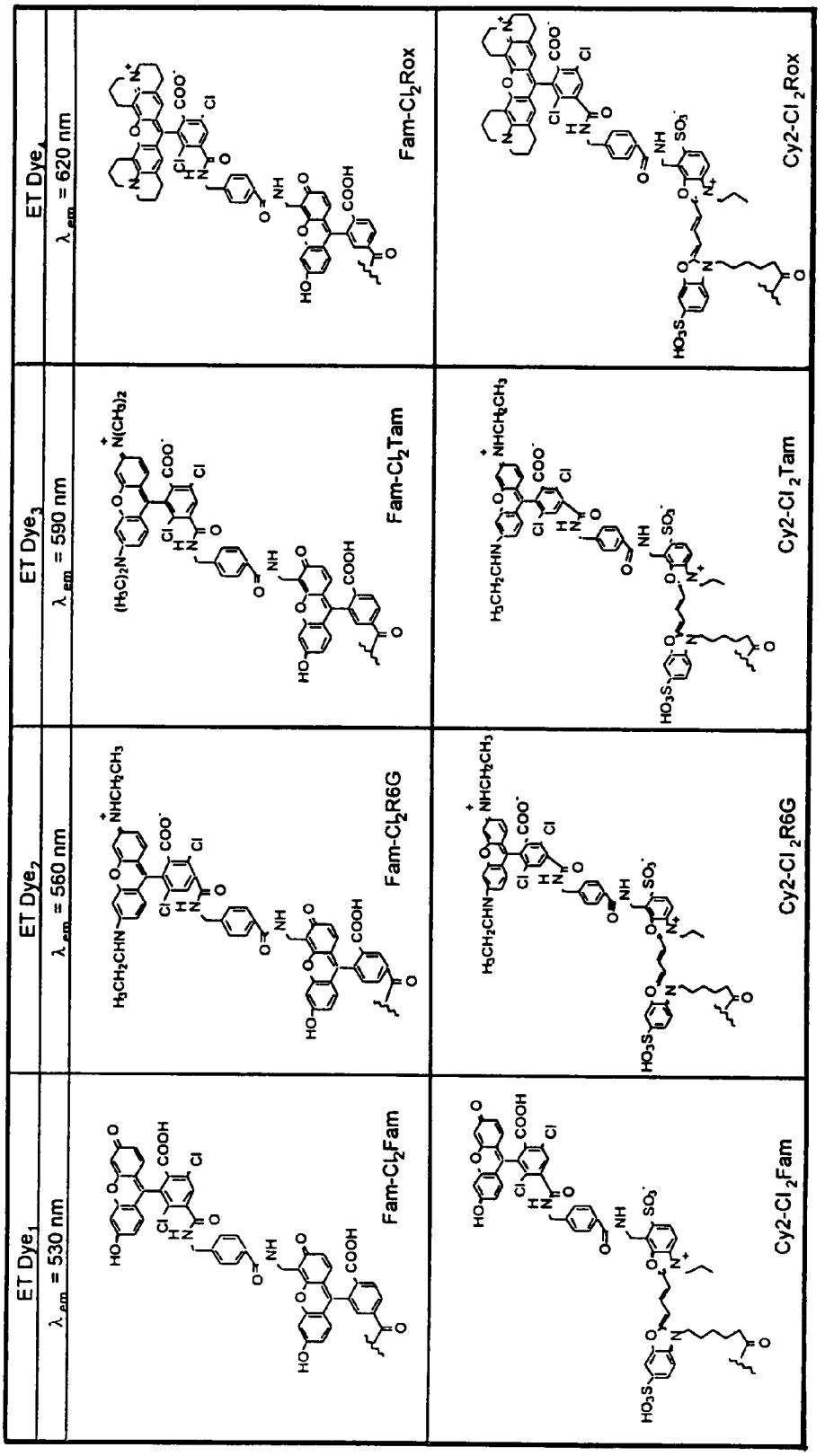

ET Dye1
λem = 530 nm
ET Dye2
λem = 560 nm
ET Dye3
λem = 590 nm
ET Dye4
λem = 620 nm
Fam-Cl2Fam
Fam-Cl2R6G
Fam-Cl2Tam
Fam-Cl2Rox
Cy2-Cl2Fam
Cy2-Cl2R6G
Cy2-Cl2Tam
Cy2-Cl2Rox

FIGURE 16

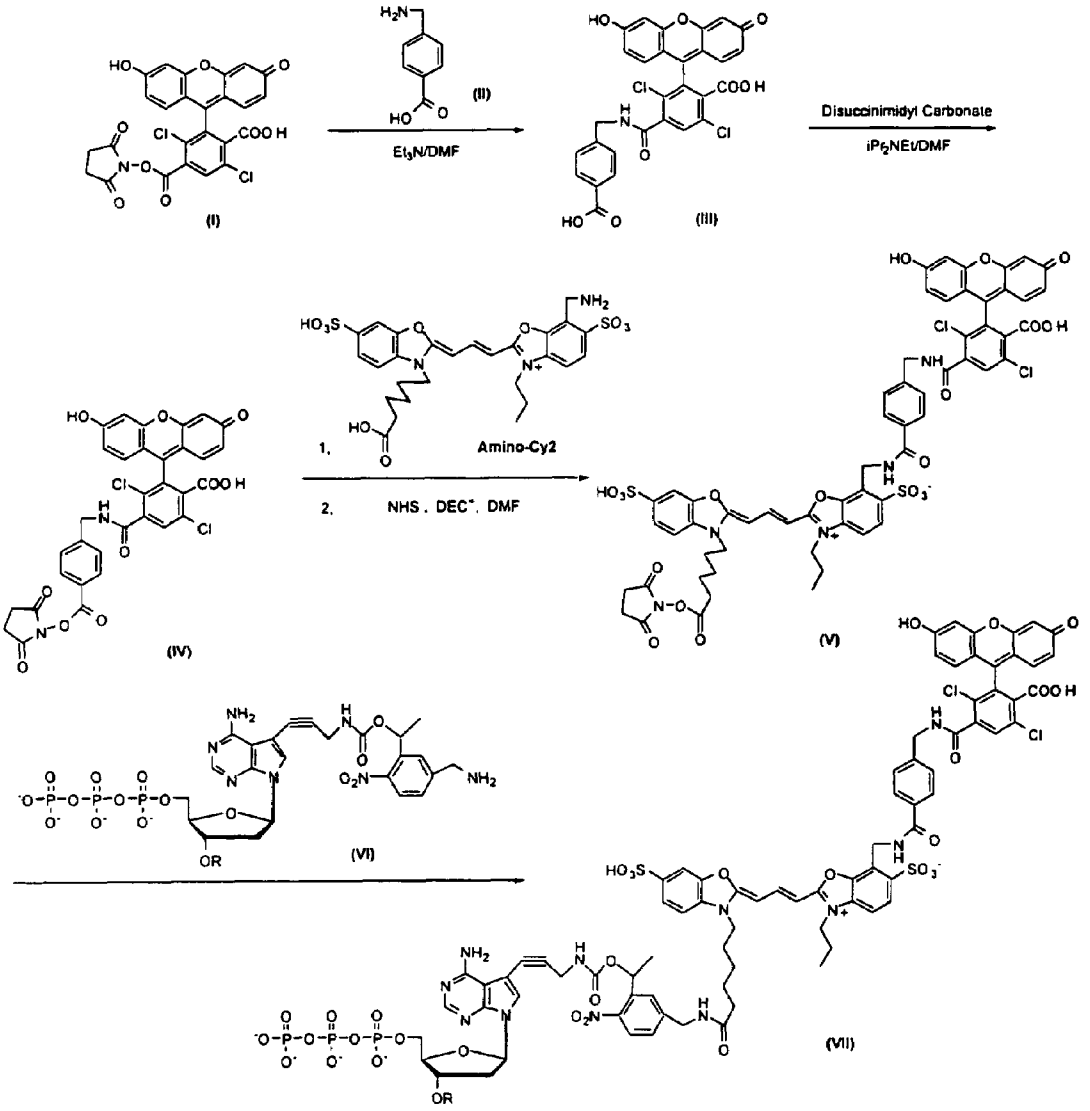
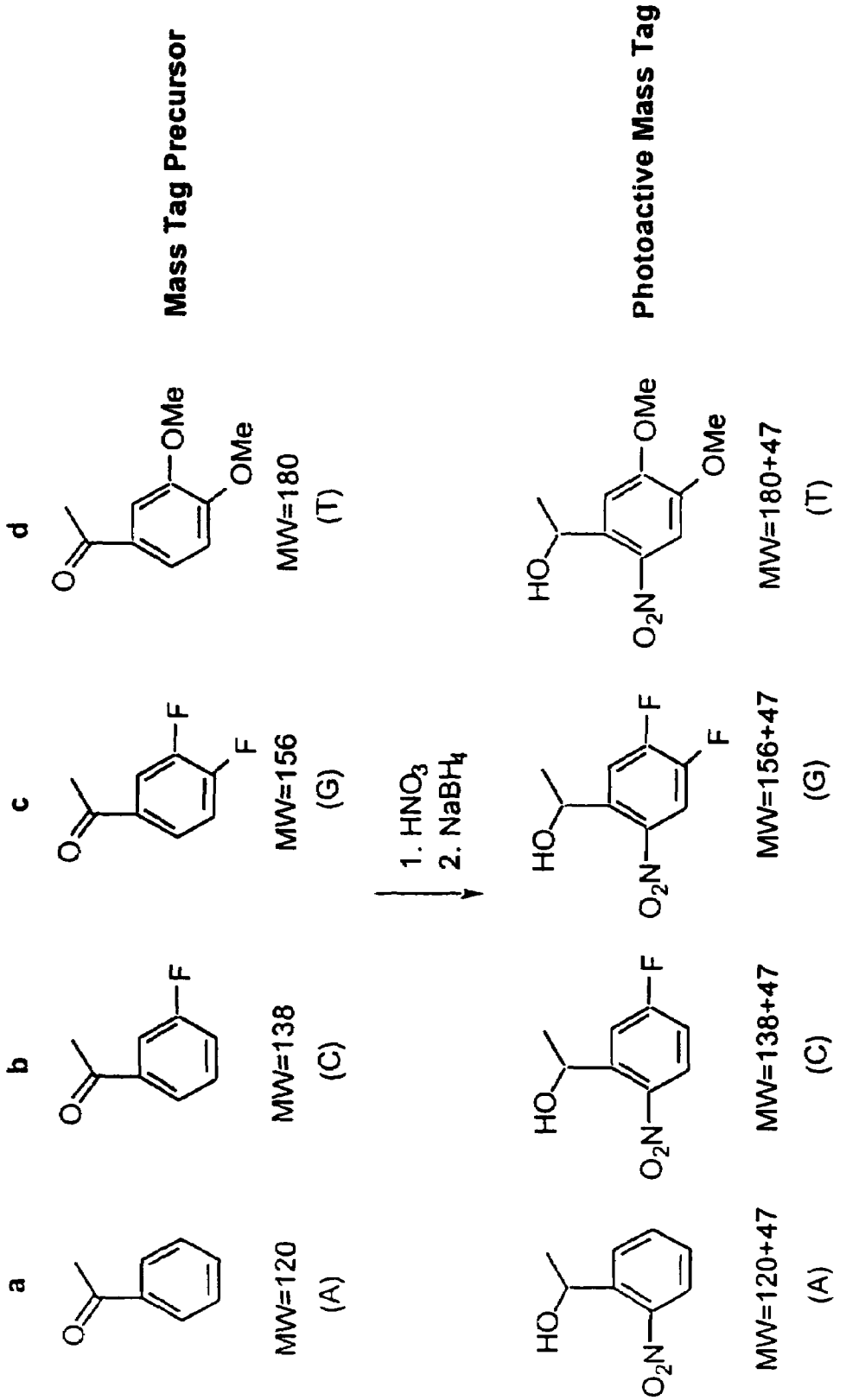

(I)
(II)
Et3N/DMF
(III)
Disuccinimidyl Carbonate
iPr2NEt/DMF
1. Amino-Cy2
2. NHS . DEC* . DMF
(IV)
(V)
(VI)
(VII)

FIGURE 17


Mass Tag Precursor
Photoactive Mass Tag
a
b
c
d
1. HNO3
2. NaBH4
MW=120
(A)
MW=138
(C)
MW=156
(G)
MW=180
(T)
MW=120+47
(A)
MW=138+47
(C)
MW=156+47
(G)
MW=180+47
(T)

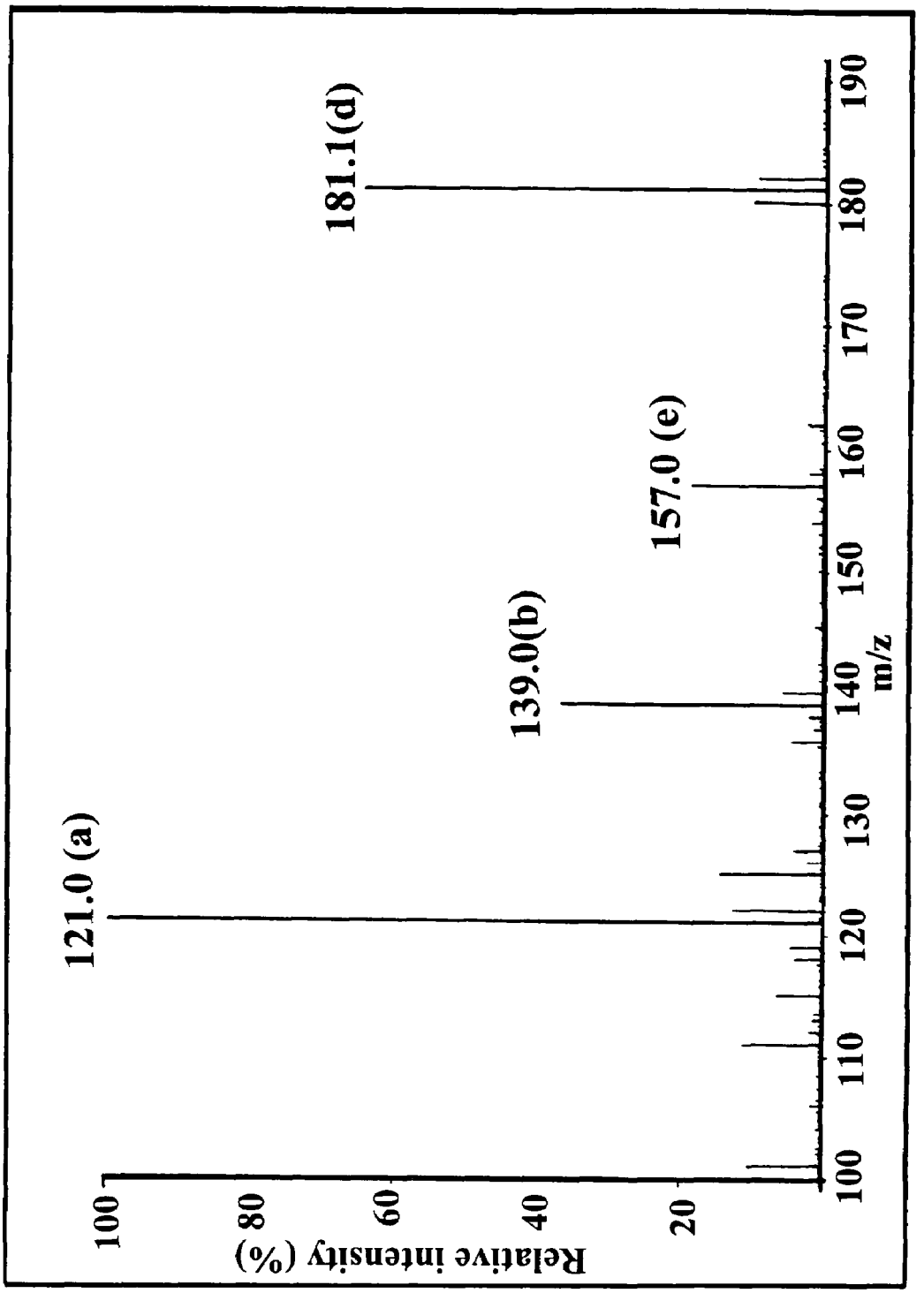
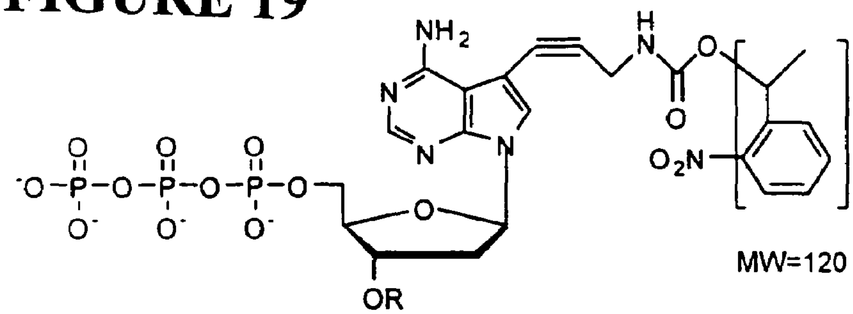
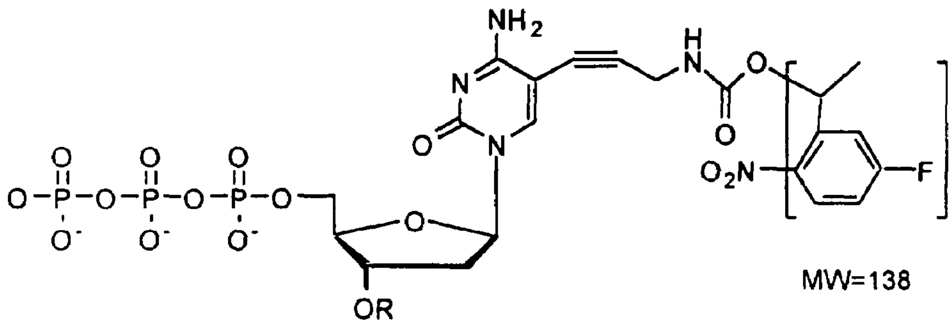

121.0 (a)
139.0(b)
157.0 (e)
181.1(d)
Relative intensity (%)
100
80
60
40
20
100
110
120
130
140
150
160
170
180
190
m/z

FIGURE 18

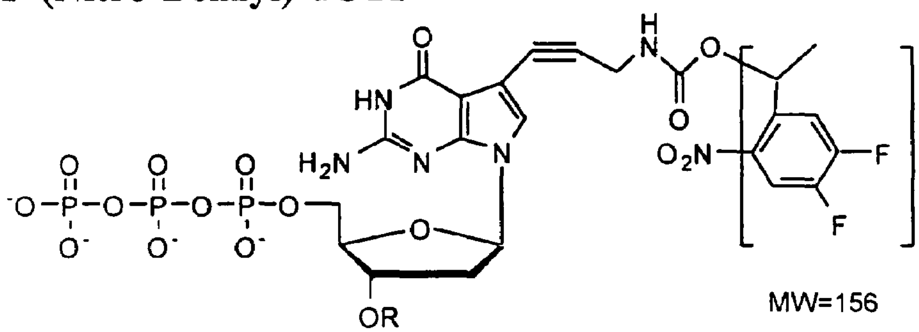
FIGURE 19



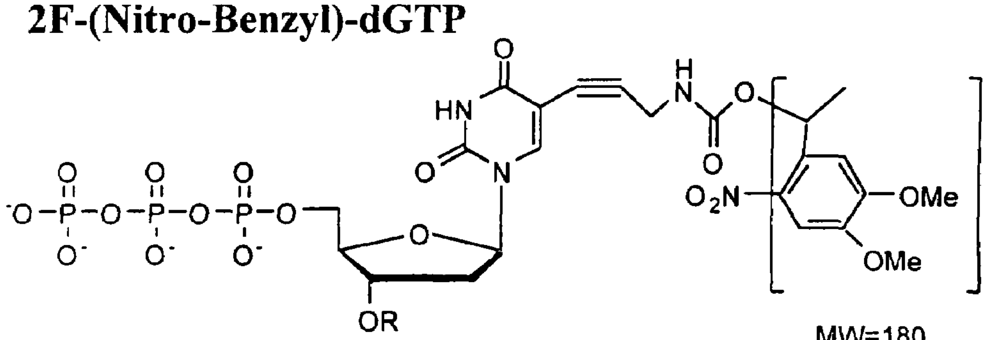
(Nitro-Benzyl)-dATP



F-(Nitro-Benzyl)-dCTP



2F-(Nitro-Benzyl)-dGTP



2(Meo)-(Nitro-Benzyl)-dTTP

R = H, MOM or Allyl

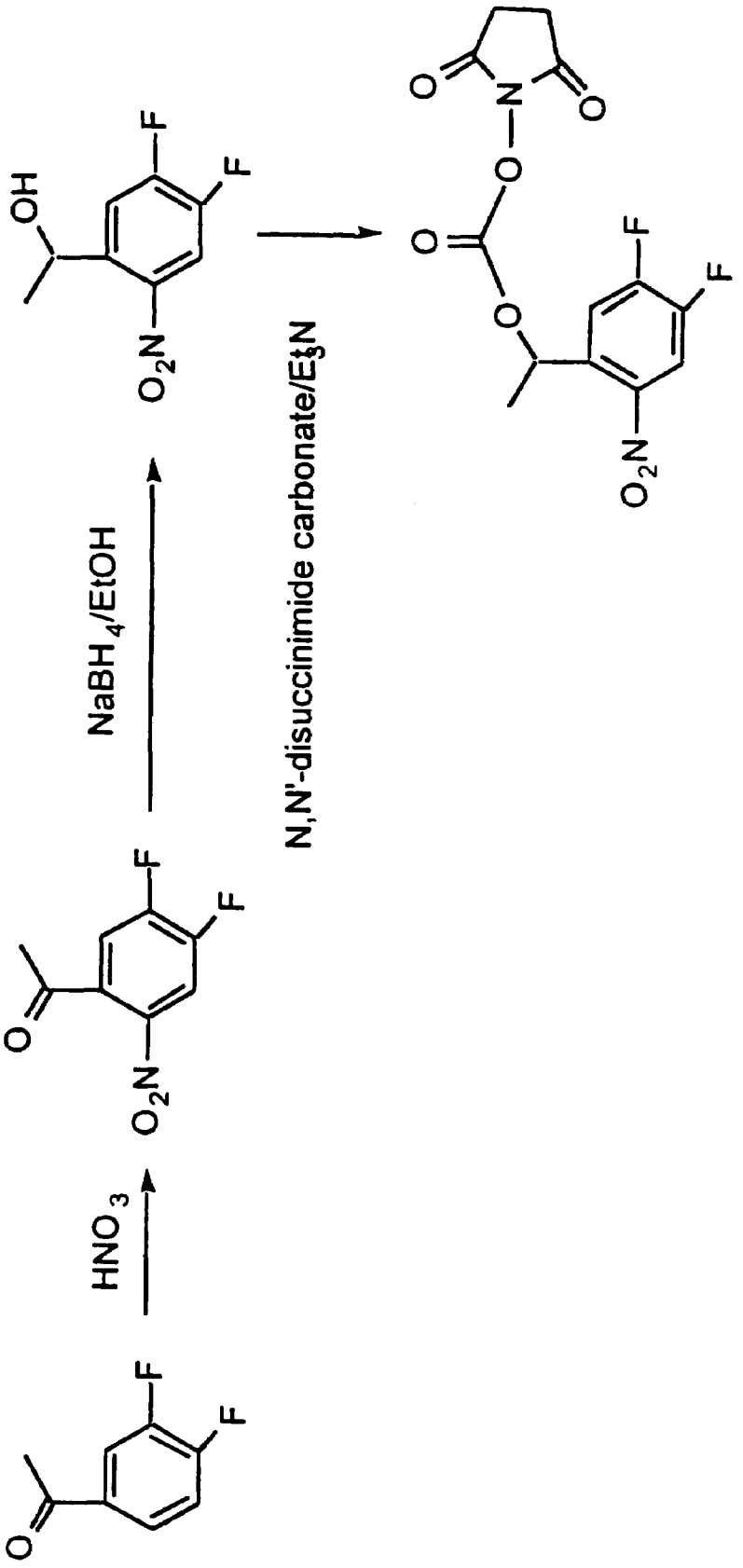


FIGURE 20

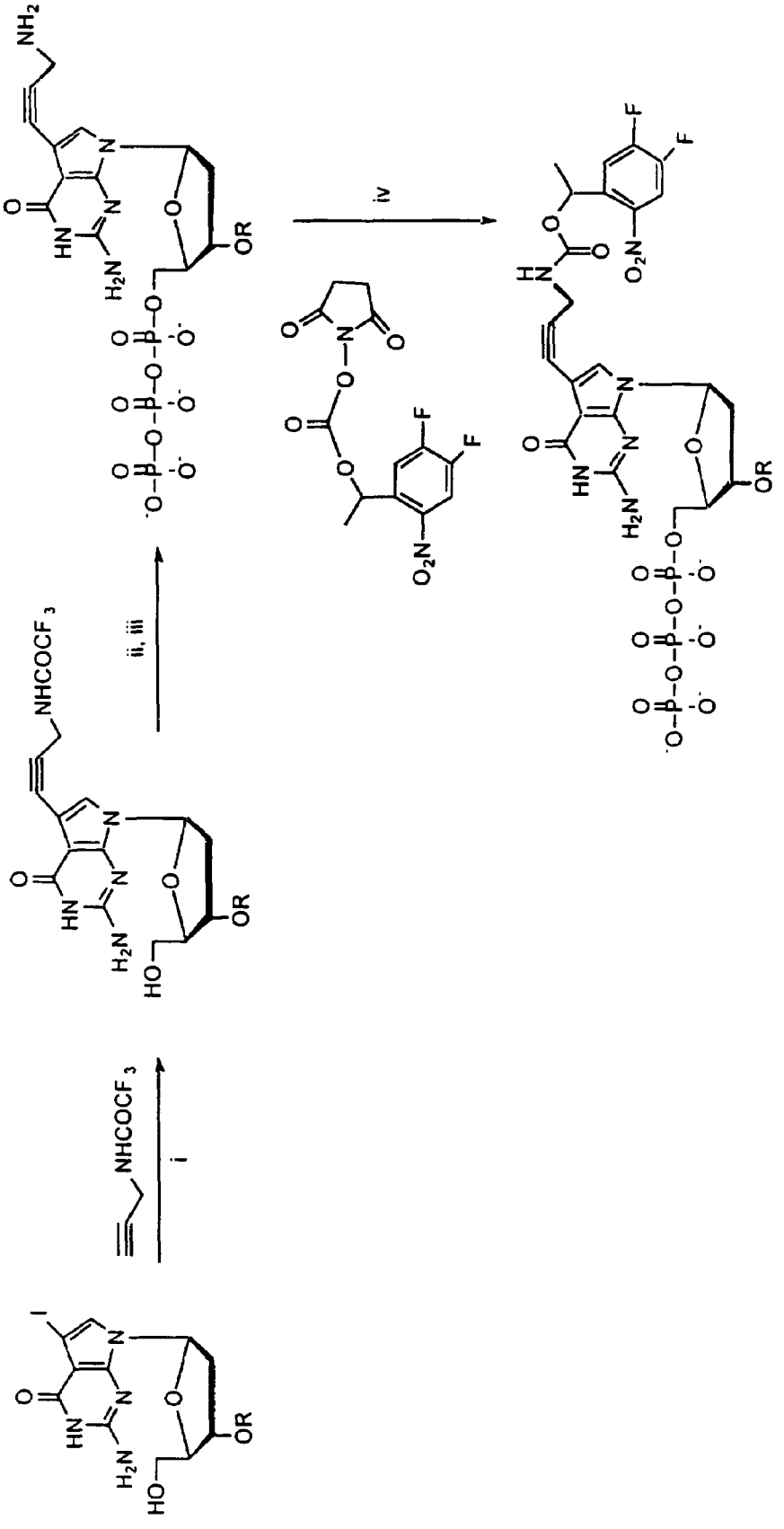
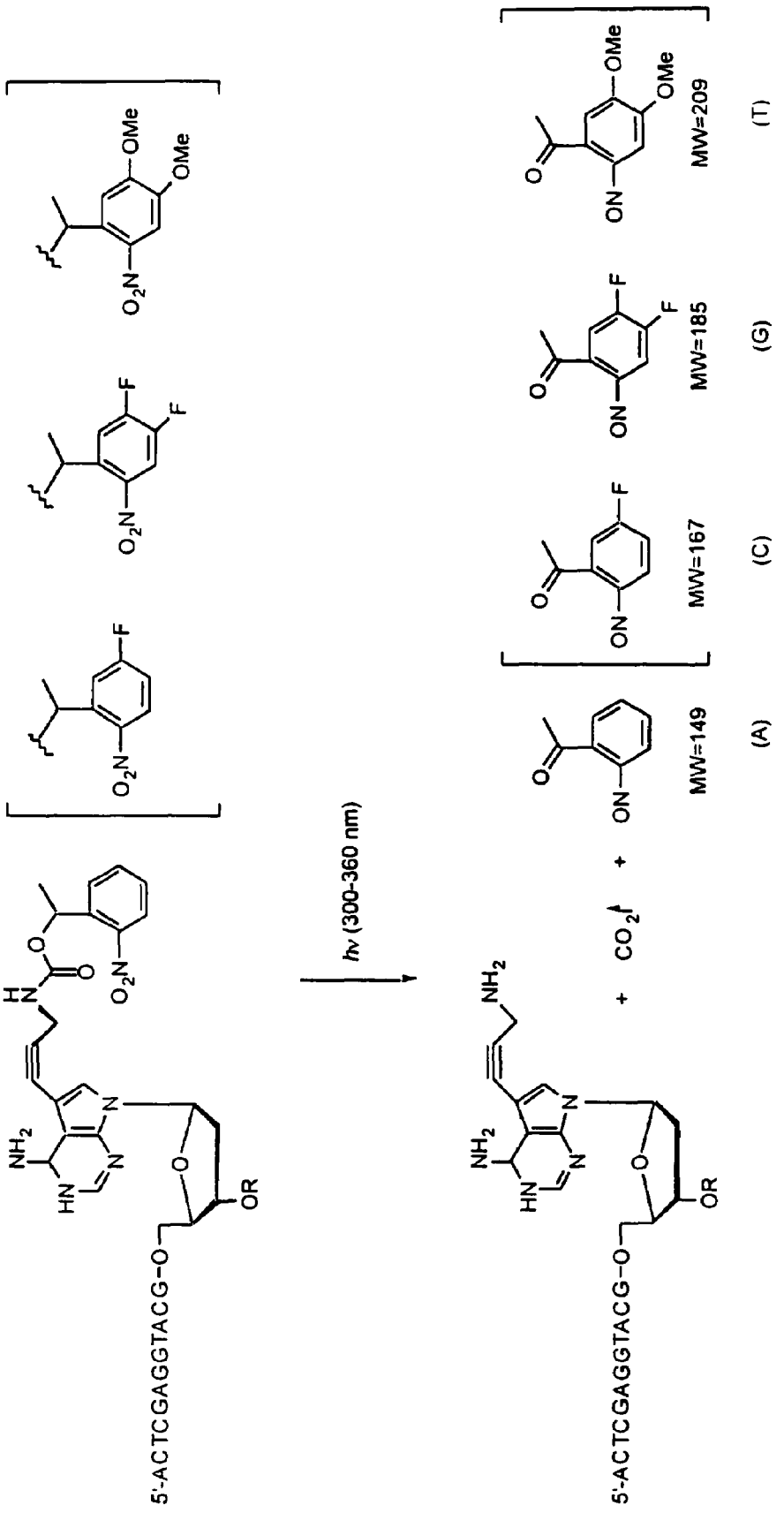


FIGURE 21

FIGURE 22


hν (300-360 nm)
5'-ACTCGAGGTACG-O
OR
+ CO2
MW=149
(A)
MW=167
(C)
MW=185
(G)
MW=209
(T)

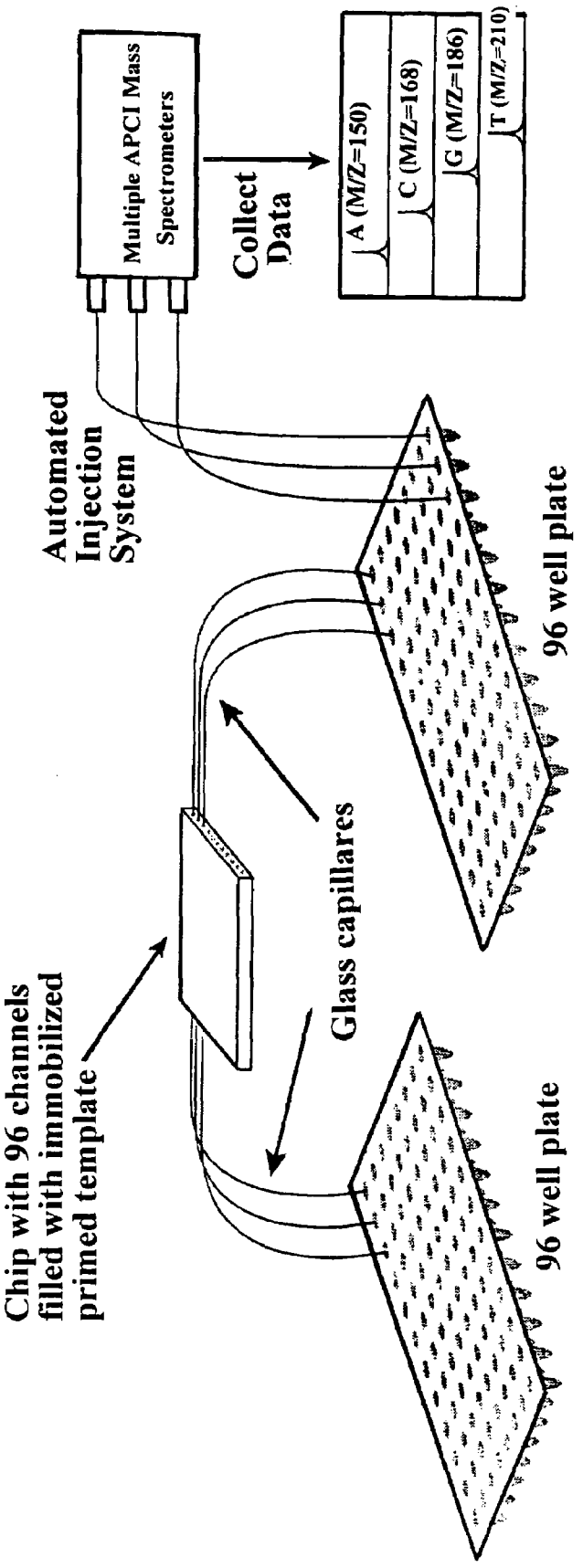

Multiple APCI Mass Spectrometers
Collect Data
A (M/Z=150)
C (M/Z=168)
G (M/Z=186)
T (M/Z=210)
Automated Injection System
96 well plate
Glass capillares
Chip with 96 channels filled with immobilized primed template
96 well plate

FIGURE 23

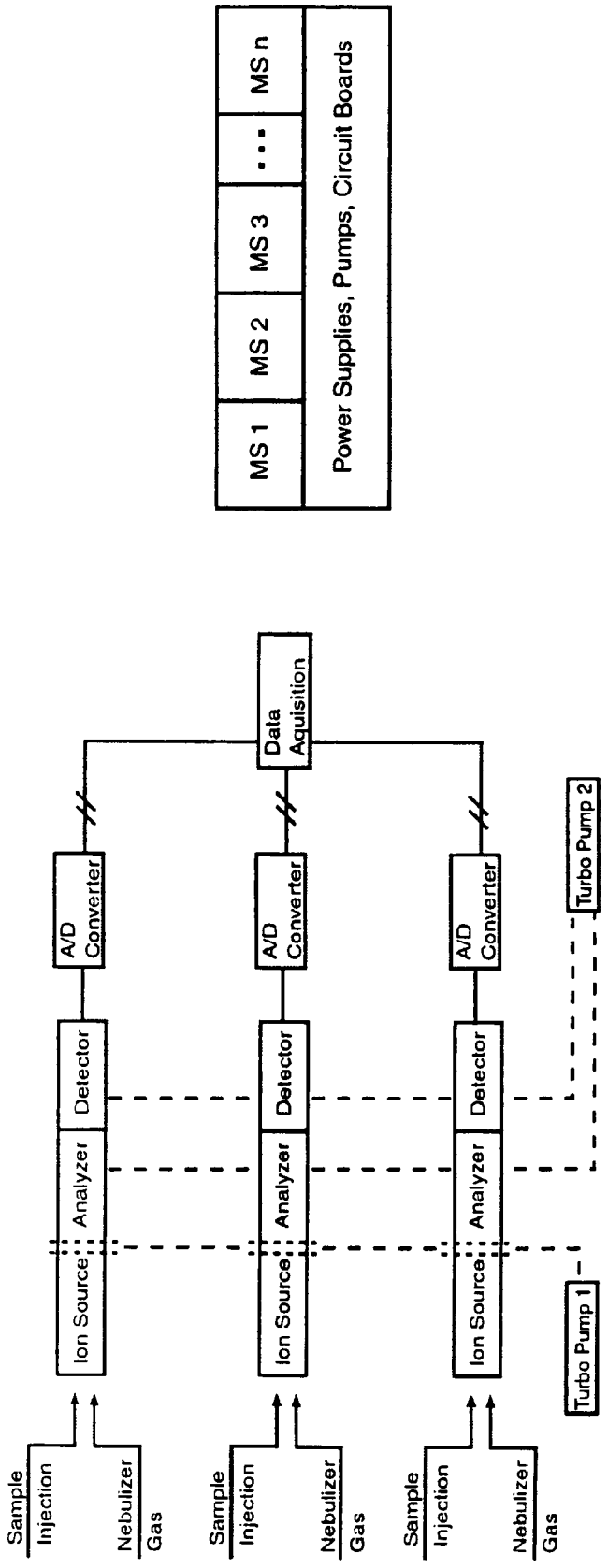

MS 1
MS 2
MS 3
...
MS n
Power Supplies, Pumps, Circuit Boards
Data Aquisition
A/D Converter
A/D Converter
A/D Converter
Detector
Detector
Detector
Analyzer
Analyzer
Analyzer
Ion Source
Ion Source
Ion Source
Turbo Pump 2
Turbo Pump 1
Sample Injection
Nebulizer Gas
Sample Injection
Nebulizer Gas
Sample Injection
Nebulizer Gas

FIGURE 24

MASSIVE PARALLEL METHOD FOR DECODING DNA AND RNA

This application is a continuation of U.S. Ser. No. 10/702,203, filed Nov. 4, 2003, now U.S. Pat. No. 7,345,159 which is a divisional of U.S. Ser. No. 09/972,364, filed Oct. 5, 2001, now U.S. Pat. No. 6,664,079, issued Dec. 16, 2003, which claims the benefit of U.S. Provisional Application No. 60/300,894, filed Jun. 26, 2001, and is a continuation-in-part of U.S. Ser. No. 09/684,670, filed Oct. 6, 2000, now abandoned, the contents of each of which are hereby incorporated by reference in their entireties into this application.

The invention disclosed herein was made with government support under National Science Foundation award no. BES0097793. Accordingly, the U.S. Government has certain rights in this invention.

BACKGROUND OF THE INVENTION

Throughout this application, various publications are referenced in parentheses by author and year. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe the state of the art to which this invention pertains.

The ability to sequence deoxyribonucleic acid (DNA) accurately and rapidly is revolutionizing biology and medicine. The confluence of the massive Human Genome Project is driving an exponential growth in the development of high throughput genetic analysis technologies. This rapid technological development involving chemistry, engineering, biology, and computer science makes it possible to move from studying single genes at a time to analyzing and comparing entire genomes.

With the completion of the first entire human genome sequence map, many areas in the genome that are highly polymorphic in both exons and introns will be known. The pharmacogenomics challenge is to comprehensively identify the genes and functional polymorphisms associated with the variability in drug response (Roses, 2000). Resequencing of polymorphic areas in the genome that are linked to disease development will contribute greatly to the understanding of diseases, such as cancer, and therapeutic development. Thus, high-throughput accurate methods for resequencing the highly variable intron/exon regions of the genome are needed in order to explore the full potential of the complete human genome sequence map. The current state-of-the-art technology for high throughput DNA sequencing, such as used for the Human Genome Project (Pennisi 2000), is capillary array DNA sequencers using laser induced fluorescence detection (Smith et al., 1986; Ju et al. 1995, 1996; Kheterpal et al. 1996; Salas-Solano et al. 1998). Improvements in the polymerase that lead to uniform termination efficiency and the introduction of thermostable polymerases have also significantly improved the quality of sequencing data (Tabor and Richardson, 1987, 1995). Although capillary array DNA sequencing technology to some extent addresses the throughput and read length requirements of large scale DNA sequencing projects, the throughput and accuracy required for mutation studies needs to be improved for a wide variety of applications ranging from disease gene discovery to forensic identification. For example, electrophoresis based DNA sequencing methods have difficulty detecting heterozygotes unambiguously and are not 100% accurate in regions rich in nucleotides comprising guanine or cytosine due to compressions (Bowling et al. 1991; Yamakawa et al. 1997). In addition, the first few bases after the priming site are often masked by the high fluorescence signal from excess dye-labeled primers or dye-labeled terminators, and are therefore difficult to identify. Therefore, the requirement of electrophoresis for DNA sequencing is still the bottleneck for high-throughput DNA sequencing and mutation detection projects.

The concept of sequencing DNA by synthesis without using electrophoresis was first revealed in 1988 (Hyman, 1988) and involves detecting the identity of each nucleotide as it is incorporated into the growing strand of DNA in a polymerase reaction. Such a scheme coupled with the chip format and laser-induced fluorescent detection has the potential to markedly increase the throughput of DNA sequencing projects. Consequently, several groups have investigated such a system with an aim to construct an ultra high-throughput DNA sequencing procedure (Cheeseman 1994, Metzker et al. 1994). Thus far, no complete success of using such a system to unambiguously sequence DNA has been reported. The pyrosequencing approach that employs four natural nucleotides (comprising a base of adenine (A), cytosine (C), guanine (G), or thymine (T)) and several other enzymes for sequencing DNA by synthesis is now widely used for mutation detection (Ronaghi 1998). In this approach, the detection is based on the pyrophosphate (PPi) released during the DNA polymerase reaction, the quantitative conversion of pyrophosphate to adenosine triphosphate (ATP) by sulfurylase, and the subsequent production of visible light by firefly luciferase. This procedure can only sequence up to 30 base pairs (bps) of nucleotide sequences, and each of the 4 nucleotides needs to be added separately and detected separately. Long stretches of the same bases cannot be identified unambiguously with the pyrosequencing method.

More recent work in the literature exploring DNA sequencing by a synthesis method is mostly focused on designing and synthesizing a photocleavable chemical moiety that is linked to a fluorescent dye to cap the 3'-OH group of deoxynucleoside triphosphates (dNTPs) (Welch et al. 1999). Limited success for the incorporation of the 3'-modified nucleotide by DNA polymerase is reported. The reason is that the 3'-position on the deoxyribose is very close to the amino acid residues in the active site of the polymerase, and the polymerase is therefore sensitive to modification in this area of the deoxyribose ring. On the other hand, it is known that modified DNA polymerases (Thermo Sequenase and Taq FS polymerase) are able to recognize nucleotides with extensive modifications with bulky groups such as energy transfer dyes at the 5-position of the pyrimidines (T and C) and at the 7-position of purines (G and A) (Rosenblum et al. 1997, Zhu et al. 1994). The ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP) have been determined (Pelletier et al. 1994) which supports this fact. As shown in FIG. **1**, the 3-D structure indicates that the surrounding area of the 3'-position of the deoxyribose ring in ddCTP is very crowded, while there is ample space for modification on the 5-position the cytidine base.

The approach disclosed in the present application is to make nucleotide analogues by linking a unique label such as a fluorescent dye or a mass tag through a cleavable linker to the nucleotide base or an analogue of the nucleotide base, such as to the 5-position of the pyrimidines (T and C) and to the 7-position of the purines (G and A), to use a small cleavable chemical moiety to cap the 3'-OH group of the deoxyribose to make it nonreactive, and to incorporate the nucleotide analogues into the growing DNA strand as terminators. Detection of the unique label will yield the sequence identity of the nucleotide. Upon removing the label and the 3'-OH

capping group, the polymerase reaction will proceed to incorporate the next nucleotide analogue and detect the next base.

It is also desirable to use a photocleavable group to cap the 3'-OH group. However, a photocleavable group is generally bulky and thus the DNA polymerase will have difficulty to incorporate the nucleotide analogues containing a photocleavable moiety capping the 3'-OH group. If small chemical moieties that can be easily cleaved chemically with high yield can be used to cap the 3'-OH group, such nucleotide analogues should also be recognized as substrates for DNA polymerase. It has been reported that 3'-O-methoxy-deoxynucleotides are good substrates for several polymerases (Axelrod et al. 1978). 3'-O-allyl-dATP was also shown to be incorporated by Ventr(exo-) DNA polymerase in the growing strand of DNA (Metzker et al. 1994). However, the procedure to chemically cleave the methoxy group is stringent and requires anhydrous conditions. Thus, it is not practical to use a methoxy group to cap the 3'-OH group for sequencing DNA by synthesis. An ester group was also explored to cap the 3'-OH group of the nucleotide, but it was shown to be cleaved by the nucleophiles in the active site in DNA polymerase (Canard et al. 1995). Chemical groups with electrophiles such as ketone groups are not suitable for protecting the 3'-OH of the nucleotide in enzymatic reactions due to the existence of strong nucleophiles in the polymerase. It is known that MOM ($—CH_2OCH_3$) and allyl ($—CH_2CH═CH_2$) groups can be used to cap an —OH group, and can be cleaved chemically with high yield (Ireland et al. 1986; Kamal et al. 1999). The approach disclosed in the present application is to incorporate nucleotide analogues, which are labeled with cleavable, unique labels such as fluorescent dyes or mass tags and where the 3'-OH is capped with a cleavable chemical moiety such as either a MOM group ($—CH_2OCH_3$) or an allyl group ($—CH_2CH═CH_2$), into the growing strand DNA as terminators. The optimized nucleotide set ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$, where R denotes the chemical group used to cap the 3'-OH) can then be used for DNA sequencing by the synthesis approach.

There are many advantages of using mass spectrometry (MS) to detect small and stable molecules. For example, the mass resolution can be as good as one dalton. Thus, compared to gel electrophoresis sequencing systems and the laser induced fluorescence detection approach which have overlapping fluorescence emission spectra, leading to heterozygote detection difficulty, the MS approach disclosed in this application produces very high resolution of sequencing data by detecting the cleaved small mass tags instead of the long DNA fragment. This method also produces extremely fast separation in the time scale of microseconds. The high resolution allows accurate digital mutation and heterozygote detection. Another advantage of sequencing with mass spectrometry by detecting the small mass tags is that the compressions associated with gel based systems are completely eliminated.

In order to maintain a continuous hybridized primer extension product with the template DNA, a primer that contains a stable loop to form an entity capable of self-priming in a polymerase reaction can be ligated to the 3' end of each single stranded DNA template that is immobilized on a solid surface such as a chip. This approach will solve the problem of washing off the growing extension products in each cycle.

Saxon and Bertozzi (2000) developed an elegant and highly specific coupling chemistry linking a specific group that contains a phosphine moiety to an azido group on the surface of a biological cell. In the present application, this coupling chemistry is adopted to create a solid surface which is coated with a covalently linked phosphine moiety, and to generate polymerase chain reaction (PCR) products that contain an azido group at the 5' end for specific coupling of the DNA template with the solid surface. One example of a solid surface is glass channels which have an inner wall with an uneven or porous surface to increase the surface area. Another example is a chip.

The present application discloses a novel and advantageous system for DNA sequencing by the synthesis approach which employs a stable DNA template, which is able to self prime for the polymerase reaction, covalently linked to a solid surface such as a chip, and 4 unique nucleotides analogues ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$). The success of this novel system will allow the development of an ultra high-throughput and high fidelity DNA sequencing system for polymorphism, pharmacogenetics applications and for whole genome sequencing. This fast and accurate DNA resequencing system is needed in such fields as detection of single nucleotide polymorphisms (SNPs) (Chee et al. 1996), serial analysis of gene expression (SAGE) (Velculescu et al. 1995), identification in forensics, and genetic disease association studies.

SUMMARY OF THE INVENTION

This invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) detecting the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the —OH group at the 3'-position of the deoxyribose to uncap the —OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (viii) so as to detect the identity of a newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and

wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose.

The invention provides a parallel mass spectrometry system, which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1**: The 3D structure of the ternary complexes of rat DNA polymerase, a DNA template-primer, and dideoxycytidine triphosphate (ddCTP). The left side of the illustration shows the mechanism for the addition of ddCTP and the right side of the illustration shows the active site of the polymerase. Note that the 3' position of the dideoxyribose ring is very crowded, while ample space is available at the 5 position of the cytidine base.

FIG. **2**A-**2**B: Scheme of sequencing by the synthesis approach. A: Example where the unique labels are dyes and the solid surface is a chip. B: Example where the unique labels are mass tags and the solid surface is channels etched into a glass chip. A, C, G, T; nucleotide triphosphates comprising bases adenine, cytosine, guanine, and thymine; d, deoxy; dd, dideoxy; R, cleavable chemical group used to cap the —OH group; Y, cleavable linker.

FIG. **3**: The synthetic scheme for the immobilization of an azido (N_3) labeled DNA fragment to a solid surface coated with a triarylphosphine moiety. Me, methyl group; P, phosphorus; Ph, phenyl.

FIG. **4**: The synthesis of triarylphosphine N-hydroxysuccinimide (NHS) ester.

FIG. **5**: The synthetic scheme for attaching an azido (N_3) group through a linker to the 5' end of a DNA fragment, which is then used to couple with the triarylphosphine moiety on a solid surface. DMSO, dimethylsulfonyl oxide.

FIG. **6**A-**6**B: Ligate the looped primer (B) to the immobilized single stranded DNA template forming a self primed DNA template moiety on a solid surface. P (in circle), phosphate.

FIG. **7**: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique fluorescent dye attached to the base through a photocleavable linker and the 3'-OH is either exposed or capped with a MOM group or an allyl group. FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **8**: A representative scheme for the synthesis of the nucleotide analogue $_{3'\text{-}RO}$-G-$_{Tam}$. A similar scheme can be used to create the other three modified nucleotides: $_{3'\text{-}RO}$-A-$_{Dye1}$, $_{3'\text{-}RO}$-C-$_{Dye2}$, $_{3'\text{-}RO}$-T-$_{Dye4}$. (i) tetrakis(triphenylphosphine)palladium(0); (ii) $POCl_3$, En_4N^+pyrophosphate; (iii) NH_4OH; (iv) $Na_2CO_3/NaHCO_3$ (pH=9.0)/DMSO.

FIG. **9**: A scheme for testing the sequencing by synthesis approach. Each nucleotide, modified by the attachment of a unique fluorescent dye, is added one by one, based on the complimentary template. The dye is detected and cleaved to test the approach. Dye1=Fam; Dye2=R6G; Dye3=Tam; Dye4=Rox.

FIG. **10**: The expected photocleavage products of DNA containing a photo-cleavable dye (Tam). Light absorption (300-360 nm) by the aromatic 2-nitrobenzyl moiety causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond located in the 2-position followed by cleavage and decarboxylation (Pillai 1980).

FIG. **11**: Synthesis of PC-LC-Biotin-FAM to evaluate the photolysis efficiency of the fluorophore coupled with the photocleavable linker 2-nitrobenzyl group.

FIG. **12**: Fluorescence spectra (λ_{ex}=480 nm) of PC-LC-Biotin-FAM immobilized on a microscope glass slide coated with streptavidin (a); after 10 min photolysis (λ_{irr}=350 nm; ~0.5 mW/cm^2) (b); and after washing with water to remove the photocleaved dye (c).

FIG. **13**A-**13**B: Synthetic scheme for capping the 3'-OH of nucleotide.

FIG. **14**: Chemical cleavage of the MOM group (top row) and the allyl group (bottom row) to free the 3'-OH in the nucleotide. CITMS=chlorotrimethylsilane.

FIG. **15**A-**15**B: Examples of energy transfer coupled dye systems, where Fam or Cy2 is employed as a light absorber (energy transfer donor) and Cl_2Fam, Cl_2R6G, Cl_2Tam, or Cl_2Rox as an energy transfer acceptor. Cy2, cyanine; FAM, 5-carboxyfluorescein; R6G, 6-carboxyrhodamine-6G; TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; ROX, 6-carboxy-X-rhodamine.

FIG. **16**: The synthesis of a photocleavable energy transfer dye-labeled nucleotide. DMF, dimethylformide. DEC=1-(3-dimethylaminopropyl)-3-ethylcarbodimide hydrochloride. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **17**: Structures of four mass tag precursors and four photoactive mass tags. Precursors: a) acetophenone; b) 3-fluoroacetophenone; c) 3,4-difluoroacetophenone; and d) 3,4-dimethoxyacetophenone. Four photoactive mass tags are used to code for the identity of each of the four nucleotides (A, C, G, T).

FIG. **18**: Atmospheric Pressure Chemical Ionization (APCI) mass spectrum of mass tag precursors shown in FIG. **17**.

FIG. **19**: Examples of structures of four nucleotide analogues for use in the sequencing by synthesis approach. Each nucleotide analogue has a unique mass tag attached to the base through a photocleavable linker, and the 3'-OH is either exposed or capped with a MOM group or an allyl group. The square brackets indicated that the mass tag is cleavable. R═H, CH_2OCH_3 (MOM) or $CH_2CH{=}CH_2$ (Allyl).

FIG. **20**: Example of synthesis of NHS ester of one mass tag (Tag-3). A similar scheme is used to create other mass tags.

FIG. **21**: A representative scheme for the synthesis of the nucleotide analogue $_{3'\text{-}RO}$-G-$_{Tag3}$. A similar scheme is used to create the other three modified bases $_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-

C-$_{Tag2}$, $_{3'\text{-}RO}$-T-$_{Tag4}$. (i) tetrakis(triphenylphosphine)palladium(0); (ii) $POCl_3$, Bn_4N^+pyrophosphate; (iii) NH_4OH; (iv) $Na_2CO_3/NaHCO_3$ (pH=9.0)/DMSO.

FIG. **22**: Examples of expected photocleavage products of DNA containing a photocleavable mass tag.

FIG. **23**: System for DNA sequencing comprising multiple channels in parallel and multiple mass spectrometers in parallel. The example shows 96 channels in a silica glass chip.

FIG. **24**: Parallel mass spectrometry system for DNA sequencing. Example shows three mass spectrometers in parallel. Samples are injected into the ion source where they are mixed with a nebulizer gas and ionized. A turbo pump is used to continuously sweep away free radicals, neutral compounds and other undesirable elements coming from the ion source. A second turbo pump is used to generate a continuous vacuum in all three analyzers and detectors simultaneously. The acquired signal is then converted to a digital signal by the A/D converter. All three signals are then sent to the data acquisition processor to convert the signal to identify the mass tag in the injected sample and thus identify the nucleotide sequence.

DETAILED DESCRIPTION OF THE INVENTION

The following definitions are presented as an aid in understanding this invention.

As used herein, to cap an —OH group means to replace the "H" in the —OH group with a chemical group. As disclosed herein, the —OH group of the nucleotide analogue is capped with a cleavable chemical group. To uncap an —OH group means to cleave the chemical group from a capped —OH group and to replace the chemical group with "H", i.e., to replace the "R" in —OR with "H" wherein "R" is the chemical group used to cap the —OH group.

The nucleotide bases are abbreviated as follows: adenine (A), cytosine (C), guanine (G), thymine (T), and uracil (U).

An analogue of a nucleotide base refers to a structural and functional derivative of the base of a nucleotide which can be recognized by polymerase as a substrate. That is, for example, an analogue of adenine (A) should form hydrogen bonds with thymine (T), a C analogue should form hydrogen bonds with G, a G analogue should form hydrogen bonds with C, and a T analogue should form hydrogen bonds with A, in a double helix format. Examples of analogues of nucleotide bases include, but are not limited to, 7-deaza-adenine and 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom.

A nucleotide analogue refers to a chemical compound that is structurally and functionally similar to the nucleotide, i.e. the nucleotide analogue can be recognized by polymerase as a substrate. That is, for example, a nucleotide analogue comprising adenine or an analogue of adenine should form hydrogen bonds with thymine, a nucleotide analogue comprising C or an analogue of C should form hydrogen bonds with G, a nucleotide analogue comprising G or an analogue of G should form hydrogen bonds with C, and a nucleotide analogue comprising T or an analogue of T should form hydrogen bonds with A, in a double helix format. Examples of nucleotide analogues disclosed herein include analogues which comprise an analogue of the nucleotide base such as 7-deaza-adenine or 7-deaza-guanine, wherein the nitrogen atom at the 7-position of adenine or guanine is substituted with a carbon atom. Further examples include analogues in which a label is attached through a cleavable linker to the 5-position of cytosine or thymine or to the 7-position of deaza-adenine or deaza-guanine. Other examples include analogues in which a small chemical moiety such as $—CH_2OCH_3$ or $—CH_2CH{=}CH_2$ is used to cap the —OH group at the 3'-position of deoxyribose. Analogues of dideoxynucleotides can similarly be prepared.

As used herein, a porous surface is a surface which contains pores or is otherwise uneven, such that the surface area of the porous surface is increased relative to the surface area when the surface is smooth.

The present invention is directed to a method for sequencing a nucleic acid by detecting the identity of a nucleotide analogue after the nucleotide analogue is incorporated into a growing strand of DNA in a polymerase reaction, which comprises the following steps:

(i) attaching a 5' end of the nucleic acid to a solid surface;

(ii) attaching a primer to the nucleic acid attached to the solid surface;

(iii) adding a polymerase and one or more different nucleotide analogues to the nucleic acid to thereby incorporate a nucleotide analogue into the growing strand of DNA, wherein the incorporated nucleotide analogue terminates the polymerase reaction and wherein each different nucleotide analogue comprises (a) a base selected from the group consisting of adenine, guanine, cytosine, thymine, and uracil, and their analogues; (b) a unique label attached through a cleavable linker to the base or to an analogue of the base; (c) a deoxyribose; and (d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose;

(iv) washing the solid surface to remove unincorporated nucleotide analogues;

(v) detecting the unique label attached to the nucleotide analogue that has been incorporated into the growing strand of DNA, so as to thereby identify the incorporated nucleotide analogue;

(vi) adding one or more chemical compounds to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on a primer extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer;

(vii) cleaving the cleavable linker between the nucleotide analogue that was incorporated into the growing strand of DNA and the unique label;

(viii) cleaving the cleavable chemical group capping the —OH group at the 3'-position of the deoxyribose to uncap the —OH group, and washing the solid surface to remove cleaved compounds; and

(ix) repeating steps (iii) through (viii) so as to detect the identity of a newly incorporated nucleotide analogue into the growing strand of DNA;

wherein if the unique label is a dye, the order of steps (v) through (vii) is: (v), (vi), and (vii); and

wherein if the unique label is a mass tag, the order of steps (v) through (vii) is: (vi), (vii), and (v).

In one embodiment of any of the nucleotide analogues described herein, the nucleotide base is adenine. In one embodiment, the nucleotide base is guanine. In one embodiment, the nucleotide base is cytosine. In one embodiment, the nucleotide base is thymine. In one embodiment, the nucleotide base is uracil. In one embodiment, the nucleotide base is an analogue of adenine. In one embodiment, the nucleotide base is an analogue of guanine. In one embodiment, the nucleotide base is an analogue of cytosine. In one embodiment, the nucleotide base is an analogue of thymine. In one embodiment, the nucleotide base is an analogue of uracil.

In different embodiments of any of the inventions described herein, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in a chip, or a porous channel in a chip. In one

embodiment, the solid surface is glass. In one embodiment, the solid surface is silicon. In one embodiment, the solid surface is gold. In one embodiments, the solid surface is a magnetic bead. In one embodiment, the solid surface is a chip. In one embodiment, the solid surface is a channel in a chip. In one embodiment, the solid surface is a porous channel in a chip. Other materials can also be used as long as the material does not interfere with the steps of the method.

In one embodiment, the step of attaching the nucleic acid to the solid surface comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to the 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

In one embodiment, the nucleic acid that is attached to the solid surface is a single-stranded deoxyribonucleic acid (DNA). In another embodiment, the nucleic acid that is attached to the solid surface in step (i) is a double-stranded DNA, wherein only one strand is directly attached to the solid surface, and wherein the strand that is not directly attached to the solid surface is removed by denaturing before proceeding to step (ii). In one embodiment, the nucleic acid that is attached to the solid surface is a ribonucleic acid (RNA), and the polymerase in step (iii) is reverse transcriptase.

In one embodiment, the primer is attached to a 3' end of the nucleic acid in step (ii), and the attached primer comprises a stable loop and an —OH group at a 3'-position of a deoxyribose capable of self-priming in the polymerase reaction. In one embodiment, the step of attaching the primer to the nucleic acid comprises hybridizing the primer to the nucleic acid or ligating the primer to the nucleic acid. In one embodiment, the primer is attached to the nucleic acid through a ligation reaction which links the 3' end of the nucleic acid with the 5' end of the primer.

In one embodiment, one or more of four different nucleotide analogs is added in step (iii), wherein each different nucleotide analogue comprises a different base selected from the group consisting of thymine or uracil or an analogue of thymine or uracil, adenine or an analogue of adenine, cytosine or an analogue of cytosine, and guanine or an analogue of guanine, and wherein each of the four different nucleotide analogues comprises a unique label.

In one embodiment, the cleavable chemical group that caps the —OH group at the 3'-position of the deoxyribose in the nucleotide analogue is $—CH_2OCH_3$ or $—CH_2CH═CH_2$. Any chemical group could be used as long as the group 1) is stable during the polymerase reaction, 2) does not interfere with the recognition of the nucleotide analogue by polymerase as a substrate, and 3) is cleavable.

In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine. In one embodiment, the fluorescent moiety is 5-carboxyfluorescein. In one embodiment, the fluorescent moiety is 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the fluorescent moiety is 6-carboxy-X-rhodamine.

In one embodiment, the unique label that is attached to the nucleotide analogue is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine. In one embodiment, the energy transfer acceptor is dichlorocarboxyfluorescein. In one embodiment, the energy transfer acceptor is dichloro-6-carboxyrhodamine-6G. In one embodiment, the energy transfer acceptor is dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine. In one embodiment, the energy transfer acceptor is dichloro-6-carboxy-X-rhodamine.

In one embodiment, the unique label that is attached to the nucleotide analogue is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group consisting of a 2-nitro-α-methyl-benzyl group, a 2-nitro-α-methyl-3-fluorobenzyl group, a 2-nitro-α-methyl-3,4-difluorobenzyl group, and a 2-nitro-α-methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-benzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3-fluorobenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3,4-difluorobenzyl group. In one embodiment, the mass tag is a 2-nitro-α-methyl-3,4-dimethoxybenzyl group. In one embodiment, the mass tag is detected using a parallel mass spectrometry system which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags.

In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

In one embodiment, the linker between the unique label and the nucleotide analogue is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical means. In one embodiment, the linker is cleaved by a chemical means. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

In one embodiment, the cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose is cleaved by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In one embodiment, the linker is cleaved by a physical chemical means. In one embodiment, the linker is cleaved by heat. In one embodiment, the linker is cleaved by light. In one embodiment, the linker is cleaved by ultraviolet light.

In one embodiment, the chemical compounds added in step (vi) to permanently cap any unreacted —OH group on the

primer attached to the nucleic acid or on the primer extension strand are a polymerase and one or more different dideoxynucleotides or analogues of dideoxynucleotides. In further embodiments, the different dideoxynucleotides are selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate, 2',3'-dideoxyguanosine 5'-triphosphate, 2',3'-dideoxycytidine 5'-triphosphate, 2',3'-dideoxythymidine 5'-triphosphate, 2',3'-dideoxyuridine 5'-triphosphase, and their analogues. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 50'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

In one embodiment, a polymerase and one or more of four different dideoxynucleotides are added in step (vi), wherein each different dideoxynucleotide is selected from the group consisting of 2',3'-dideoxyadenosine 5'-triphosphate or an analogue of 2',3'-dideoxyadenosine 5'-triphosphate; 2',3'-dideoxyguanosine 5'-triphosphate or an analogue of 2',3'-dideoxyguanosine 5'-triphosphate; 2',3'-dideoxycytidine 5'-triphosphate or an analogue of 2',3'-dideoxycytidine 5'-triphosphate; and 2',3'-dideoxythymidine 5'-triphosphate or 2',3'-dideoxyuridine 5'-triphosphase or an analogue of 2',3'-dideoxythymidine 5'-triphosphate or an analogue of 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyadenosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyguanosine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxycytidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is 2',3'-dideoxyuridine 5'-triphosphase. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxythymidine 5'-triphosphate. In one embodiment, the dideoxynucleotide is an analogue of 2',3'-dideoxyuridine 5'-triphosphase.

Another type of chemical compound that reacts specifically with the —OH group could also be used to permanently cap any unreacted —OH group on the primer attached to the nucleic acid or on an extension strand formed by adding one or more nucleotides or nucleotide analogues to the primer.

The invention provides a method for simultaneously sequencing a plurality of different nucleic acids, which comprises simultaneously applying any of the methods disclosed herein for sequencing a nucleic acid to the plurality of different nucleic acids. In different embodiments, the method can be used to sequence from one to over 100,000 different nucleic acids simultaneously.

The invention provides for the use of any of the methods disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

The invention provides a method of attaching a nucleic acid to a solid surface which comprises:

(i) coating the solid surface with a phosphine moiety,

(ii) attaching an azido group to a 5' end of the nucleic acid, and

(iii) immobilizing the 5' end of the nucleic acid to the solid surface through interaction between the phosphine moiety on the solid surface and the azido group on the 5' end of the nucleic acid.

In one embodiment, the step of coating the solid surface with the phosphine moiety comprises:

(i) coating the surface with a primary amine, and

(ii) covalently coupling a N-hydroxysuccinimidyl ester of triarylphosphine with the primary amine.

In different embodiments, the solid surface is glass, silicon, or gold. In different embodiments, the solid surface is a magnetic bead, a chip, a channel in an chip, or a porous channel in a chip.

In different embodiments, the nucleic acid that is attached to the solid surface is a single-stranded or double-stranded DNA or a RNA. In one embodiment, the nucleic acid is a double-stranded DNA and only one strand is attached to the solid surface. In a further embodiment, the strand of the double-stranded DNA that is not attached to the solid surface is removed by denaturing.

The invention provides for the use of any of the methods disclosed herein for attaching a nucleic acid to a surface for gene expression analysis, microarray based gene expression analysis, or mutation detection, translational analysis, transcriptional analysis, or for other genetic applications.

The invention provides a nucleotide analogue which comprises:

(a) a base selected from the group consisting of adenine or an analogue of adenine, cytosine or an analogue of cytosine, guanine or an analogue of guanine, thymine or an analogue of thymine, and uracil or an analogue of uracil;

(b) a unique label attached through a cleavable linker to the base or to an analogue of the base;

(c) a deoxyribose; and

(d) a cleavable chemical group to cap an —OH group at a 3'-position of the deoxyribose.

In one embodiment of the nucleotide analogue, the cleavable chemical group that caps the —OH group at the 3'-position of the deoxyribose is $—CH_2OCH_3$ or $—CH_2CH═CH_2$.

In one embodiment, the unique label is a fluorescent moiety or a fluorescent semiconductor crystal. In further embodiments, the fluorescent moiety is selected from the group consisting of 5-carboxyfluorescein, 6-carboxyrhodamine-6G, N,N,N',N'-tetramethyl-6-carboxyrhodamine, and 6-carboxy-X-rhodamine.

In one embodiment, the unique label is a fluorescence energy transfer tag which comprises an energy transfer donor and an energy transfer acceptor. In further embodiments, the energy transfer donor is 5-carboxyfluorescein or cyanine, and wherein the energy transfer acceptor is selected from the group consisting of dichlorocarboxyfluorescein, dichloro-6-carboxyrhodamine-6G, dichloro-N,N,N',N'-tetramethyl-6-carboxyrhodamine, and dichloro-6-carboxy-X-rhodamine.

In one embodiment, the unique label is a mass tag that can be detected and differentiated by a mass spectrometer. In further embodiments, the mass tag is selected from the group

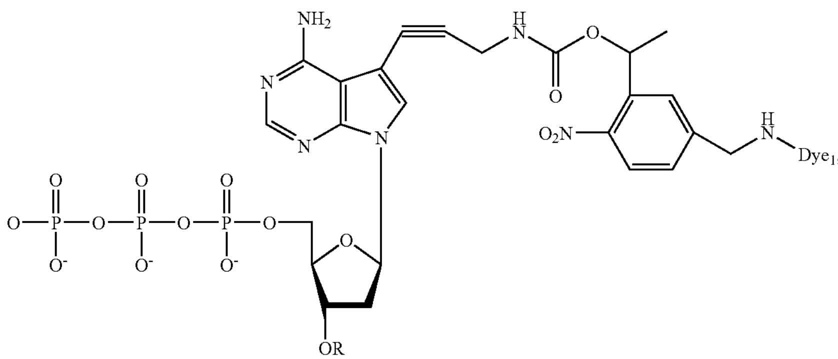
consisting of a 2-nitro-α-methyl-benzyl group, a 2-nitro-α-methyl-3-fluorobenzyl group, a 2-nitro-α-methyl-3,4-difluorobenzyl group, and a 2-nitro-α-methyl-3,4-dimethoxybenzyl group.

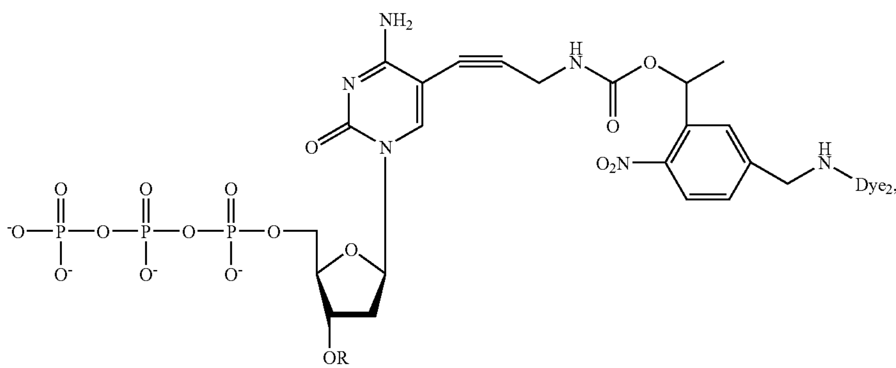
In one embodiment, the unique label is attached through a cleavable linker to a 5-position of cytosine or thymine or to a 7-position of deaza-adenine or deaza-guanine. The unique label could also be attached through a cleavable linker to another position in the nucleotide analogue as long as the attachment of the label is stable during the polymerase reaction and the nucleotide analog can be recognized by polymerase as a substrate. For example, the cleavable label could be attached to the deoxyribose.

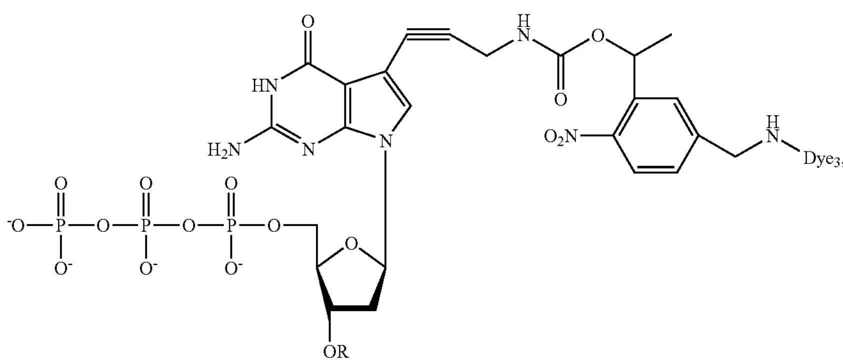
In one embodiment, the linker between the unique label and the nucleotide analogue is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light. In a further embodiment, the cleavable linker is a photocleavable linker which comprises a 2-nitrobenzyl moiety.

In one embodiment, the cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose is cleavable by a means selected from the group consisting of one or more of a physical means, a chemical means, a physical chemical means, heat, and light.

In different embodiments, the nucleotide analogue is selected from the group consisting of:







and

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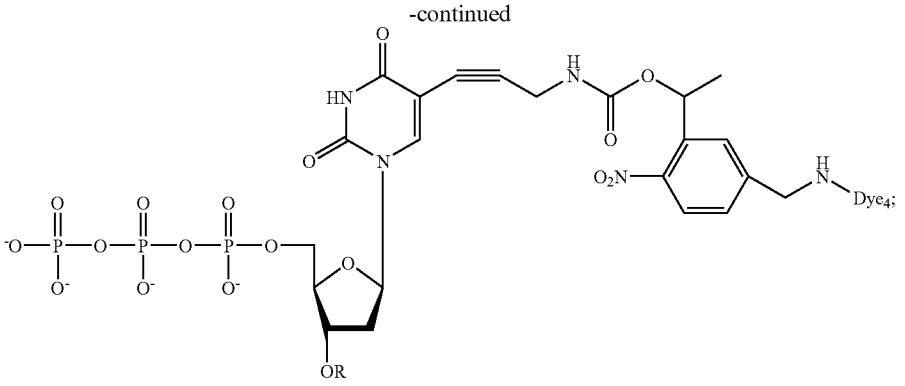
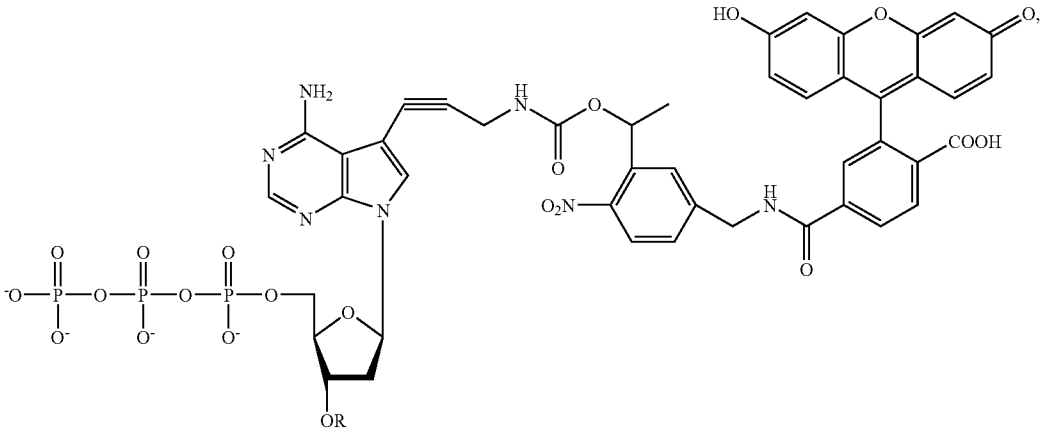
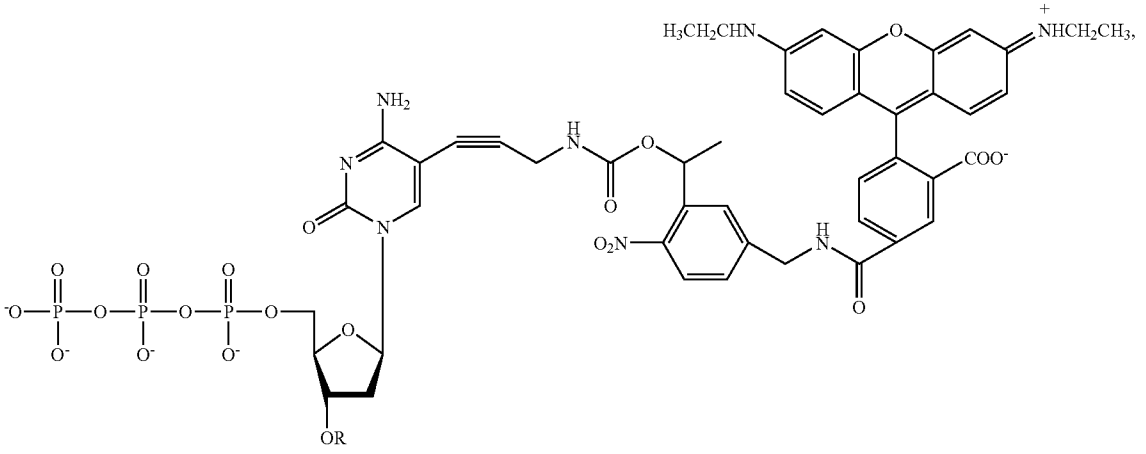


wherein Dye_1, Dye_2, Dye_3, and Dye_4 are four different unique labels; and

wherein R is a cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose.

In different embodiments, the nucleotide analogue is selected from the group consisting of:





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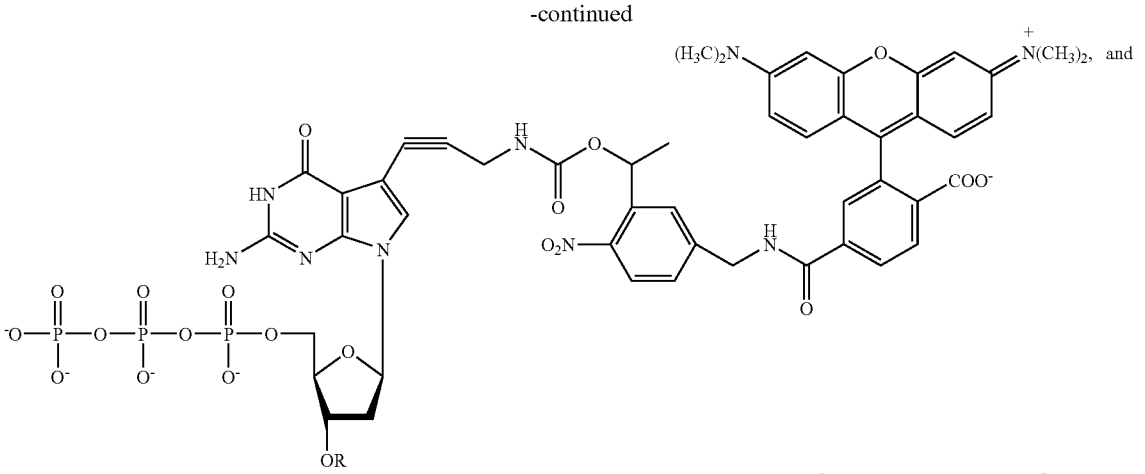
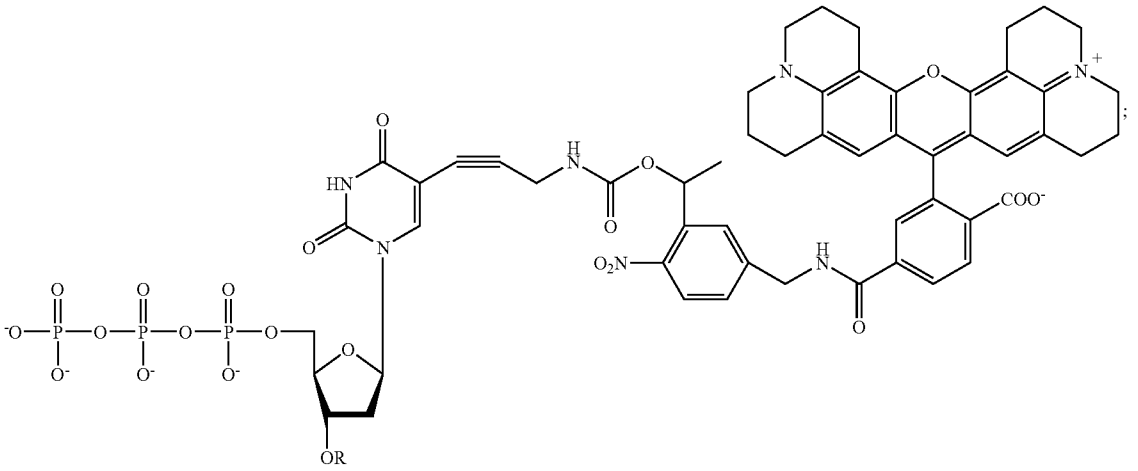
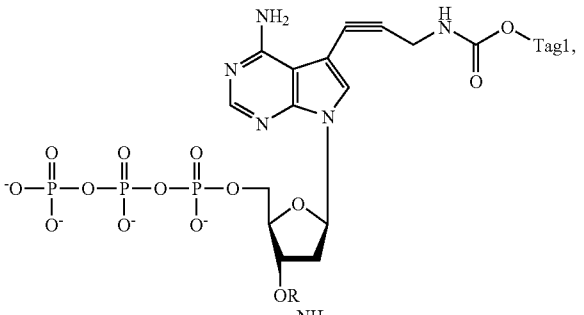
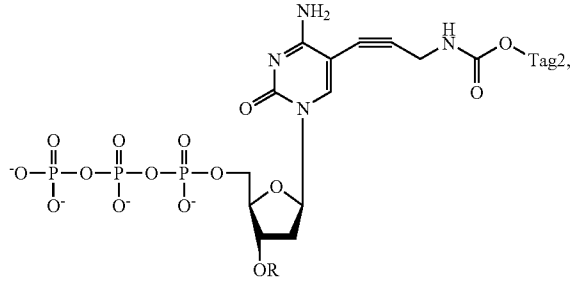




wherein R is —CH_2OCH_3 or —$CH_2CH{=}CH_2$.

In different embodiments, the nucleotide analogue is selected from the group consisting of:





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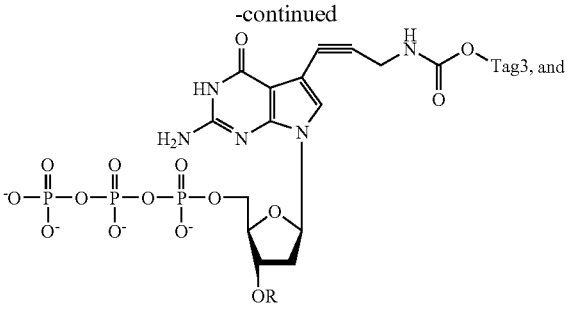
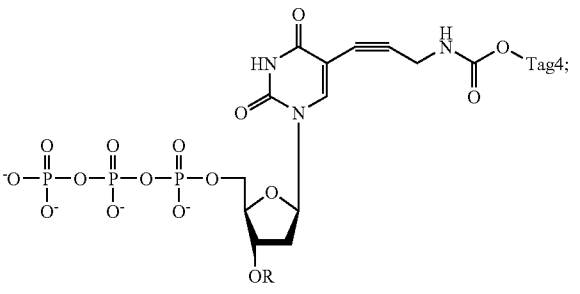
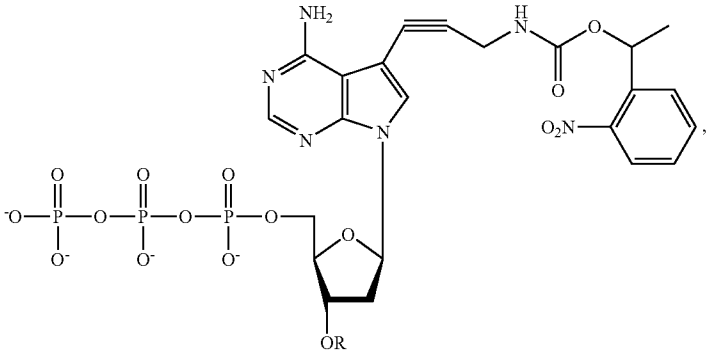


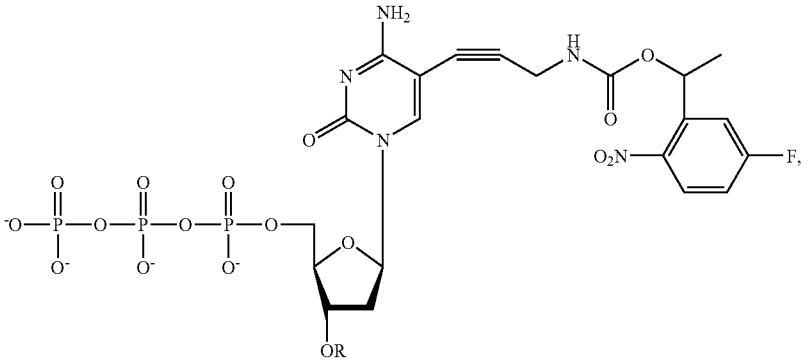


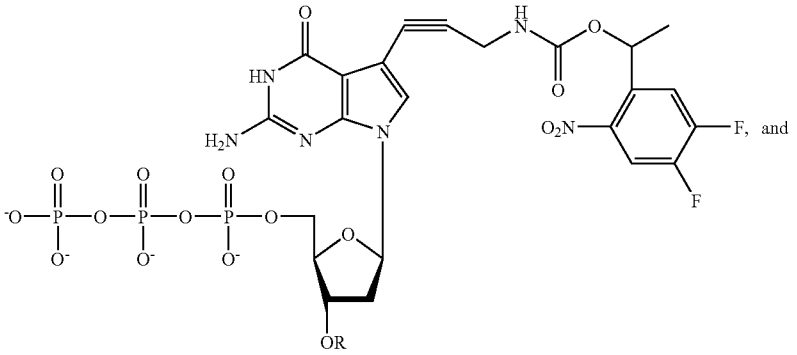
wherein Tag_1, Tag_2, Tag_3, and Tag_4 are four different mass tag labels; and

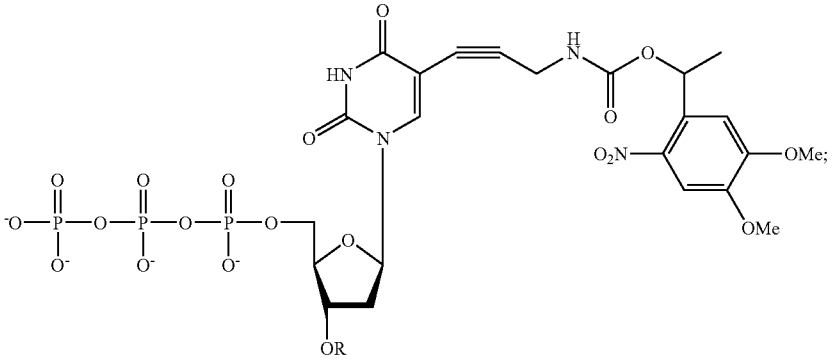
wherein R is a cleavable chemical group used to cap the —OH group at the 3'-position of the deoxyribose.

In different embodiments, the nucleotide analogue is selected from the group consisting of:









wherein R is $—CH_2OCH_3$ or $—CH_2CH═CH_2$.

The invention provides for the use any of the nucleotide analogues disclosed herein for detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

The invention provides a parallel mass spectrometry system, which comprises a plurality of atmospheric pressure chemical ionization mass spectrometers for parallel analysis of a plurality of samples comprising mass tags. In one embodiment, the mass spectrometers are quadrupole mass

spectrometers. In one embodiment, the mass spectrometers are time-of-flight mass spectrometers. In one embodiment, the mass spectrometers are contained in one device. In one embodiment, the system further comprises two turbo-pumps, wherein one pump is used to generate a vacuum and a second pump is used to remove undesired elements. In one embodiment, the system comprises at least three mass spectrometers. In one embodiment, the mass tags have molecular weights between 150 daltons and 250 daltons. The invention provides for the use of the system for DNA sequencing analysis, detection of single nucleotide polymorphisms, genetic mutation analysis, serial analysis of gene expression, gene expression analysis, identification in forensics, genetic disease association studies, DNA sequencing, genomic sequencing, translational analysis, or transcriptional analysis.

This invention will be better understood from the Experimental Details which follow. However, one skilled in the art will readily appreciate that the specific methods and results discussed are merely illustrative of the invention as described more fully in the claims which follow thereafter.

Experimental Details

1. The Sequencing by Synthesis Approach

Sequencing DNA by synthesis involves the detection of the identity of each nucleotide as it is incorporated into the growing strand of DNA in the polymerase reaction. The fundamental requirements for such a system to work are: (1) the availability of 4 nucleotide analogues (aA, aC, aG, aT) each labeled with a unique label and containing a chemical moiety capping the 3'-OH group; (2) the 4 nucleotide analogues (aA, aC, aG, aT) need to be efficiently and faithfully incorporated by DNA polymerase as terminators in the polymerase reaction; (3) the tag and the group capping the 3'-OH need to be removed with high yield to allow the incorporation and detection of the next nucleotide; and (4) the growing strand of DNA should survive the washing, detection and cleavage processes to remain annealed to the DNA template.

The sequencing by synthesis approach disclosed herein is illustrated in FIG. **2**A-**2**B. In FIG. **2**A, an example is shown where the unique labels are fluorescent dyes and the surface is a chip; in FIG. **2**B, the unique labels are mass tags and the surface is channels etched into a chip. The synthesis approach uses a solid surface such as a glass chip with an immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues ($_{3'\text{-}RO}$-A-$_{LABEL1}$, $_{3'\text{-}RO}$-C-$_{LABEL2}$, $_{3'\text{-}RO}$-G-$_{LABEL3}$, $_{3'\text{-}RO}$-T-$_{LABEL4}$) each labeled with a unique label, e.g. a fluorescent dye or a mass tag, at a specific location on the purine or pyrimidine base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by the polymerase on each spot of the surface (step 1 in FIGS. **2**A and **2**B).

As shown in FIG. **2**A, where the unique labels are dyes, after removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, a detector is used to detect the unique label. For example, a four color fluorescence imager is used to image the surface of the chip, and the unique fluorescence emission from a specific dye on the nucleotide analogues on each spot of the chip will reveal the identity of the incorporated nucleotide (step 2 in FIG. **2**A). After imaging, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess dideoxynucleoside triphosphates (ddNTPs) (ddATP, ddGTP, ddTTP, and ddCTP) and DNA polymerase to avoid interference with the next round of synthesis (step 3 in FIG. **2**A), a concept similar to the capping step in automated solid phase DNA synthesis (Caruthers, 1985). The ddNTPs, which lack a 3'-hydroxyl group, are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the dye-labeled nucleotides, and the excellent efficiency with which they are incorporated by DNA polymerase. The dye moiety is then cleaved by light (~350 nm), and the R group protecting the 3'-OH is removed chemically to generate free 3'-OH group with high yield (step 4 in FIG. **2**A). A washing step is applied to wash away the cleaved dyes and the R group. The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (step 5 in FIG. **2**A).

It is a routine procedure now to immobilize high density (>10,000 spots per chip) single stranded DNA on a 4 cm×1 cm glass chip (Schena et al. 1995). Thus, in the DNA sequencing system disclosed herein, more than 10,000 bases can be identified after each cycle and after 100 cycles, a million base pairs will be generated from one sequencing chip.

Possible DNA polymerases include Thermo Sequenase, Taq FS DNA polymerase, T7 DNA polymerase, and Vent (exo-) DNA polymerase. The fluorescence emission from each specific dye can be detected using a fluorimeter that is equipped with an accessory to detect fluorescence from a glass slide. For large scale evaluation, a multi-color scanning system capable of detecting multiple different fluorescent dyes (500 nm-700 nm) (GSI Lumonics ScanArray 5000 Standard Biochip Scanning System) on a glass slide can be used.

An example of the sequencing by synthesis approach using mass tags is shown in FIG. **2**B. The approach uses a solid surface, such as a porous silica glass channels in a chip, with immobilized DNA template that is able to self prime for initiating the polymerase reaction, and four nucleotide analogues ($_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-C-$_{Tag2}$, $_{3'\text{-}RO}$-G-$_{Tag3}$, $_{3'\text{-}RO}$-T-$_{Tag4}$) each labeled with a unique photocleavable mass tag on the specific location of the base, and a small cleavable chemical group (R) to cap the 3'-OH group. Upon adding the four nucleotide analogues and DNA polymerase, only one nucleotide analogue that is complementary to the next nucleotide on the template is incorporated by polymerase in each channel of the glass chip (step 1 in FIG. **2**B). After removing the excess reagents and washing away any unincorporated nucleotide analogues on the chip, the small amount of unreacted 3'-OH group on the self-primed template moiety is capped by excess ddNTPs (ddATP, ddGTP, ddTTP and ddCTP) and DNA polymerase to avoid interference with the next round of synthesis (step 2 in FIG. **2**B). The ddNTPs are chosen to cap the unreacted 3'-OH of the nucleotide due to their small size compared with the labeled nucleotides, and their excellent efficiency to be incorporated by DNA polymerase. The mass tags are cleaved by irradiation with light (~350 nm) (step 3 in FIG. **2**B) and then detected with a mass spectrometer. The unique mass of each tag yields the identity of the nucleotide in each channel (step 4 in FIG. **2**B). The R protecting group is then removed chemically and washed away to generate free 3'-OH group with high yield (step 5 in FIG. **2**B). The self-primed DNA moiety on the chip at this stage is ready for the next cycle of the reaction to identify the next nucleotide sequence of the template DNA (step 6 in FIG. **2**B).

Since the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), mass spectrometry has become an indispensable tool in many areas of biomedical research. Though these ionization methods are suitable for the analysis

of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation are required for implementation of mass spectrometry for DNA sequencing applications. Since the approach disclosed herein uses small and stable mass tags, there is no need to detect large DNA sequencing fragments directly and it is not necessary to use MALDI or ESI methods for detection. Atmospheric pressure chemical ionization (APCI) is an ionization method that uses a gas-phase ion-molecular reaction at atmospheric pressure (Dizidic et al. 1975). In this method, samples are introduced by either chromatography or flow injection into a pneumatic nebulizer where they are converted into small droplets by a high-speed beam of nitrogen gas. When the heated gas and solution arrive at the reaction area, the excess amount of solvent is ionized by corona discharge. This ionized mobile phase acts as the ionizing agent toward the samples and yields pseudo molecular $(M+H)^+$ and $(M-H)^-$ ions. Due to the corona discharge ionization method, high ionization efficiency is attainable, maintaining stable ionization conditions with detection sensitivity lower than femtomole region for small and stable organic compounds. However, due to the limited detection of large molecules, ESI and MALDI have replaced APCI for analysis of peptides and nucleic acids. Since in the approach disclosed the mass tags to be detected are relatively small and very stable organic molecules, the ability to detect large biological molecules gained by using ESI and MALDI is not necessary. APCI has several advantages over ESI and MALDI because it does not require any tedious sample preparation such as desalting or mixing with matrix to prepare crystals on a target plate. In ESI, the sample nature and sample preparation conditions (i.e. the existence of buffer or inorganic salts) suppress the ionization efficiency. MALDI requires the addition of matrix prior to sample introduction into the mass spectrometer and its speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. These limitations are overcome by APCI because the mass tag solution can be injected directly with no additional sample purification or preparation into the mass spectrometer. Since the mass tagged samples are volatile and have small mass numbers, these compounds are easily detectable by APCI ionization with high sensitivity. This system can be scaled up into a high throughput operation.

Each component of the sequencing by synthesis system is described in more detail below.

2. Construction of a Surface Containing Immobilized Self-primed DNA Moiety

The single stranded DNA template immobilized on a surface is prepared according to the scheme shown in FIG. **3**. The surface can be, for example, a glass chip, such as a 4 cm×1 cm glass chip, or channels in a glass chip. The surface is first treated with 0.5 M NaOH, washed with water, and then coated with high density 3-aminopropyltrimethoxysilane in aqueous ethanol (Woolley et al. 1994) forming a primary amine surface. N-Hydroxy Succinimidyl (NHS) ester of triarylphosphine (1) is covalently coupled with the primary amine group converting the amine surface to a novel triarylphosphine surface, which specifically reacts with DNA containing an azido group (2) forming a chip with immobilized DNA. Since the azido group is only located at the 5' end of the DNA and the coupling reaction is through the unique reaction of the triarylphosphine moiety with the azido group in aqueous solution (Saxon and Bertozzi 2000), such a DNA surface will provide an optimal condition for hybridization.

The NHS ester of triarylphosphine (1) is prepared according to the scheme shown in FIG. **4**. 3-diphenylphosphino-4-methoxycarbonyl-benzoic acid (3) is prepared according to the procedure described by Bertozzi et al. (Saxon and Bertozzi 2000). Treatment of (3) with N-Hydroxysuccinimide forms the corresponding NHS ester (4). Coupling of (4) with an amino carboxylic acid moiety produces compound (5) that has a long linker (n=1 to 10) for optimized coupling with DNA on the surface. Treatment of (5) with N-Hydroxysuccinimide generates the NHS ester (1) which is ready for coupling with the primary amine coated surface (FIG. **3**).

The azido labeled DNA (2) is synthesized according to the scheme shown in FIG. **5**. Treatment of ethyl ester of 5-bromovaleric acid with sodium azide and then hydrolysis produces 5-azidovaleric acid (Khoukhi et al., 1987), which is subsequently converted to a NHS ester for coupling with an amino linker modified oligonucleotide primer. Using the azido-labeled primer to perform polymerase chain reaction (PCR) reaction generates azido-labeled DNA template (2) for coupling with the triarylphosphine-modified surface (FIG. **3**).

The self-primed DNA template moiety on the sequencing chip is constructed as shown in FIG. **6** (A & B) using enzymatic ligation. A 5'-phosphorylated, 3'-OH capped loop oligonucleotide primer (B) is synthesized by a solid phase DNA synthesizer. Primer (B) is synthesized using a modified C phosphoramidite whose 3'-OH is capped with either a MOM ($—CH_2OCH_3$) group or an allyl ($—CH_2CH═CH_2$) group (designated by "R" in FIG. **6**) at the 3'-end of the oligonucleotide to prevent the self ligation of the primer in the ligation reaction. Thus, the looped primer can only ligate to the 3'-end of the DNA templates that are immobilized on the sequencing chip using T4 RNA ligase (Zhang et al. 1996) to form the self-primed DNA template moiety (A). The looped primer (B) is designed to contain a very stable loop (Antao et al. 1991) and a stem containing the sequence of M13 reverse DNA sequencing primer for efficient priming in the polymerase reaction once the primer is ligated to the immobilized DNA on the sequencing chip and the 3'-OH cap group is chemically cleaved off (Ireland et al. 1986; Kamal et al. 1999).

3. Sequencing by Synthesis Evaluation Using Nucleotide Analogues $_{3'\text{-}HO}$-A-$_{Dye1}$, $_{3'\text{-}HO}$-C-$_{Dye2}$, $_{3'\text{-}HO}$-G-$_{Dye3}$, $_{3'\text{-}HO}$-T-$_{Dye4}$

A scheme has been developed for evaluating the photocleavage efficiency using different dyes and testing the sequencing by synthesis approach. Four nucleotide analogues $_{3'\text{-}HO}$-A-$_{Dye1}$, $_{3'\text{-}HO}$-C-$_{Dye2}$, $_{3'\text{-}HO}$-G-$_{Dye3}$, $_{3'\text{-}HO}$-T-$_{Dye4}$ each labeled with a unique fluorescent dye through a photocleavable linker are synthesized and used in the sequencing by synthesis approach. Examples of dyes include, but are not limited to: Dye1=FAM, 5-carboxyfluorescein; Dye2=R6G, 6-carboxyrhodamine-6G; Dye3=TAM, N,N,N',N'-tetramethyl-6-carboxyrhodamine; and Dye4=ROX, 6-carboxy-X-rhodamine. The structures of the 4 nucleotide analogues are shown in FIG. **7** (R═H).

The photocleavable 2-nitrobenzyl moiety has been used to link biotin to DNA and protein for efficient removal by UV light (~350 nm) (Olejnik et al. 1995, 1999). In the approach disclosed herein the 2-nitrobenzyl group is used to bridge the fluorescent dye and nucleotide together to form the dye labeled nucleotides as shown in FIG. **7**.

As a representative example, the synthesis of $_{3'\text{-}HO}$-G-$_{Dye3}$ (Dye3=Tam) is shown in FIG. **8**. 7-deaza-alkynylamino-dGTP is prepared using well-established procedures (Prober et al. 1987; Lee et al. 1992 and Hobbs et al. 1991). Linker-

Tam is synthesized by coupling the Photocleavable Linker (Rollaf 1982) with NHS-Tam. 7-deaza-alkynylamino-dGTP is then coupled with the Linker-Tam to produce $_{3'\text{-}HO}$-G-$_{TAM}$. The nucleotide analogues with a free 3'-OH (i.e., R═H) are good substrates for the polymerase. An immobilized DNA template is synthesized (FIG. **9**) that contains a portion of nucleotide sequence ACGTACGACGT (SEQ ID NO: 1) that has no repeated sequences after the priming site. $_{3'\text{-}HO}$-A-$_{Dye1}$ and DNA polymerase are added to the self-primed DNA moiety and it is incorporated to the 3' site of the DNA. Then the steps in FIG. **2**A are followed (the chemical cleavage step is not required here because the 3'-OH is free) to detect the fluorescent signal from Dye-1 at 520 nm. Next, $_{3'\text{-}HO}$-C-$_{Dye2}$ is added to image the fluorescent signal from Dye-2 at 550 nm. Next, $_{3'\text{-}HO}$-G-$_{Dye3}$ is added to image the fluorescent signal from Dye-3 at 580 nm, and finally 3, $_{3'\text{-}HO}$-T-$_{Dye4}$ is added to image the fluorescent signal from Dye-4 at 610 nm.

Results on Photochemical Cleavage Efficiency

The expected photolysis products of DNA containing a photocleavable fluorescent dye at the 3' end of the DNA are shown in FIG. **10**. The 2-nitrobenzyl moiety has been successfully employed in a wide range of studies as a photocleavable-protecting group (Pillai 1980). The efficiency of the photocleavage step depends on several factors including the efficiency of light absorption by the 2-nitrobenzyl moiety, the efficiency of the primary photochemical step, and the efficiency of the secondary thermal processes which lead to the final cleavage process (Turro 1991). Burgess et al. (1997) have reported the successful photocleavage of a fluorescent dye attached through a 2-nitrobenzyl linker on a nucleotide moiety, which shows that the fluorescent dye is not quenching the photocleavage process. A photoliable protecting group based on the 2-nitrobenzyl chromophore has also been developed for biological labeling applications that involve photocleavage (Olejnik et al. 1999). The protocol disclosed herein is used to optimize the photocleavage process shown in FIG. **10**. The absorption spectra of 2-nitro benzyl compounds are examined and compared quantitatively to the absorption spectra of the fluorescent dyes. Since there will be a one-to-one relationship between the number of 2-nitrobenzyl moieties and the dye molecules, the ratio of extinction coefficients of these two species will reflect the competition for light absorption at specific wavelengths. From this information, the wavelengths at which the 2-nitrobenzyl moieties absorbed most competitively can be determined, similar to the approach reported by Olejnik et al. (1995).

A photolysis setup can be used which allows a high throughput of monochromatic light from a 1000 watt high pressure xenon lamp (LX1000UV, ILC) in conjunction with a monochromator (Kratos, Schoeffel Instruments). This instrument allows the evaluation of the photocleavage of model systems as a function of the intensity and excitation wavelength of the absorbed light. Standard analytical analysis is used to determine the extent of photocleavage. From this information, the efficiency of the photocleavage as a function of wavelength can be determined. The wavelength at which photocleavage occurs most efficiently can be selected as for use in the sequencing system.

Photocleavage results have been obtained using a model system as shown in FIG. **11**. Coupling of PC-LC-Biotin-NHS ester (Pierce, Rockford Ill.) with 5-(aminoacetamido)-fluorescein (5-aminoFAM) (Molecular Probes, Eugene Oreg.) in dimethylsulfonyl oxide (DMSO)/$NaHCO_3$ (pH=8.2) overnight at room temperature produces PC-LC-Biotin-FAM which is composed of a biotin at one end, a photocleavable 2-nitrobenzyl group in the middle, and a dye tag (FAM) at the other end. This photocleavable moiety closely mimics the designed photocleavable nucleotide analogues shown in FIG. **10**. Thus the successful photolysis of the PC-LC-Biotin-FAM moiety provides proof of the principle of high efficiency photolysis as used in the DNA sequencing system. For photolysis study, PC-LC-Biotin-FAM is first immobilized on a microscope glass slide coated with streptavidin (XENOPORE, Hawthorne N.J.). After washing off the non-immobilized PC-LC-Biotin-FAM, the fluorescence emission spectrum of the immobilized PC-LC-Biotin-FAM was taken as shown in FIG. **12** (Spectrum a). The strong fluorescence emission indicates that PC-LC-Biotin-FAM is successfully immobilized to the streptavidin coated slide surface. The photocleavability of the 2-nitrobenzyl linker by irradiation at 350 nm was then tested. After 10 minutes of photolysis (λ_{irr}=350 nm; ~0.5 mW/cm^2) and before any washing, the fluorescence emission spectrum of the same spot on the slide was taken that showed no decrease in intensity (FIG. **12**, Spectrum b), indicating that the dye (FAM) was not bleached during the photolysis process at 350 nm. After washing the glass slide with HPLC water following photolysis, the fluorescence emission spectrum of the same spot on the slide showed significant intensity decrease (FIG. **12**, Spectrum c) which indicates that most of the fluorescence dye (FAM) was cleaved from the immobilized biotin moiety and was removed by the washing procedure. This experiment shows that high efficiency cleavage of the fluorescent dye can be obtained using the 2-nitrobenzyl photocleavable linker.

4. Sequencing by Synthesis Evaluation Using Nucleotide Analogues $_{3'\text{-}RO}$-A-$_{Dye1}$, $_{3'\text{-}RO}$-C-$_{Dye2}$, $_{3'\text{-}RO}$-G-$_{Dye3}$, $_{3'\text{-}RO}$-T-$_{Dye4}$

Once the steps and conditions in Section 3 are optimized, the synthesis of nucleotide analogues $_{3'\text{-}RO}$-A-$_{Dye1}$, $_{3'\text{-}RO}$-C-$_{Dye2}$, $_{3'\text{-}RO}$-G-$_{Dye3}$, $_{3'\text{-}RO}$-T-$_{Dye4}$ can be pursued for further study of the system. Here the 3'-OH is capped in all four nucleotide analogues, which then can be mixed together with DNA polymerase and used to evaluate the sequencing system using the scheme in FIG. **9**. The MOM (—CH_2OCH_3) or allyl (—CH_2CH═CH_2) group is used to cap the 3'-OH group using well-established synthetic procedures (FIG. **13**) (Fuji et al. 1975, Metzker et al. 1994). These groups can be removed chemically with high yield as shown in FIG. **14** (Ireland, et al. 1986; Kamal et al. 1999). The chemical cleavage of the MOM and allyl groups is fairly mild and specific, so as not to degrade the DNA template moiety. For example, the cleavage of the allyl group takes 3 minutes with more than 93% yield (Kamal et al. 1999), while the MOM group is reported to be cleaved with close to 100% yield (Ireland, et al. 1986).

5. Using Energy Transfer Coupled Dyes to Optimize the Sequencing by Synthesis System

The spectral property of the fluorescent tags can be optimized by using energy transfer (ET) coupled dyes. The ET primer and ET dideoxynucleotides have been shown to be a superior set of reagents for 4-color DNA sequencing that allows the use of one laser to excite multiple sets of fluorescent tags (Ju et al. 1995). It has been shown that DNA polymerase (Thermo Sequenase and Taq FS) can efficiently incorporate the ET dye labeled dideoxynucleotides (Rosenblum et al. 1997). These ET dye-labeled sequencing reagents are now widely used in large scale DNA sequencing projects, such as the human genome project. A library of ET dye labeled nucleotide analogues can be synthesized as shown in FIG. **15** for

optimization of the DNA sequencing system. The ET dye set (FAM-Cl_2FAM, FAM-Cl_2R6G, FAM-Cl_2TAM, FAM-Cl_2ROX) using FAM as a donor and dichloro(FAM, R6G, TAM, ROX) as acceptors has been reported in the literature (Lee et al. 1997) and constitutes a set of commercially available DNA sequencing reagents. These ET dye sets have been proven to produce enhanced fluorescence intensity, and the nucleotides labeled with these ET dyes at the 5-position of T and C and the 7-position of G and A are excellent substrates of DNA polymerase. Alternatively, an ET dye set can be constructed using cyanine (Cy2) as a donor and Cl_2FAM, Cl_2R6G, Cl_2TAM, or Cl_2ROX as energy acceptors. Since Cy2 possesses higher molar absorbance compared with the rhodamine and fluorescein derivatives, an ET system using Cy2 as a donor produces much stronger fluorescence signals than the system using FAM as a donor (Hung et al. 1996). FIG. **16** shows a synthetic scheme for an ET dye labeled nucleotide analogue with Cy2 as a donor and Cl_2FAM as an acceptor using similar coupling chemistry as for the synthesis of an energy transfer system using FAM as a donor (Lee et al. 1997). Coupling of Cl_2FAM (I) with spacer 4-aminomethylbenzoic acid (II) produces III, which is then converted to NHS ester IV. Coupling of IV with amino-Cy2, and then converting the resulting compound to a NHS ester produces V, which subsequently couples with amino-photolinker nucleotide VI yields the ET dye labeled nucleotide VII.

6. Sequencing by Synthesis Evaluation Using Nucleotide Analogues $_{3'\text{-}HO}$-A-$_{Tag1}$, $_{3'\text{-}HO}$-C-$_{Tag2}$, $_{3'\text{-}HO}$-G-$_{Tag3}$, $_{3'\text{-}HO}$-T-$_{Tag4}$

The precursors of four examples of mass tags are shown in FIG. **17**. The precursors are: (a) acetophenone; (b) 3-fluoroacetophenone; (c) 3,4-difluoroacetophenone; and (d) 3,4-dimethoxyacetophenone. Upon nitration and reduction, four photoactive tags are produced from the four precursors and used to code for the identity of each of the four nucleotides (A, C, G, T). Clean APCI mass spectra are obtained for the four mass tag precursors (a, b, c, d) as shown in FIG. **18**. The peak with m/z of 121 is a, 139 is b, 157 is c, and 181 is d. This result shows that these four mass tags are extremely stable and produce very high resolution data in an APCI mass spectrometer with no cross talk between the mass tags. In the examples shown below, each of the unique m/z from each mass tag translates to the identity of the nucleotide [Tag-1 (m/z,150)= A; Tag-2 (m/z,168)=C; Tag-3 (m/z,186)=G; Tag-4 (m/z, 210)= T].

Different combinations of mass tags and nucleotides can be used, as indicated by the general scheme: $_{3'\text{-}HO}$-A-$_{Tag1}$, $_{3'\text{-}HO}$-C-$_{Tag2}$, $_{3'\text{-}HO}$-G-$_{Tag3}$, $_{3'\text{-}HO}$-T-$_{Tag4}$ where Tag1, Tag2, Tag3, and Tag4 are four different unique cleavable mass tags. Four specific examples of nucleotide analogues are shown in FIG. **19**. In FIG. **19**, "R" is H when the 3'-OH group is not capped. As discussed above, the photo cleavable 2-nitro benzyl moiety has been used to link biotin to DNA and protein for efficient removal by UV light (~350 nm) irradiation (Olejnik et al. 1995, 1999). Four different 2-nitro benzyl groups with different molecular weights as mass tags are used to form the mass tag labeled nucleotides as shown in FIG. **19**: 2-nitro-α-methyl-benzyl (Tag-1) codes for A; 2-nitro-α-methyl-3-fluorobenzyl (Tag-2) codes for C; 2-nitro-α-methyl-3,4-difluorobenzyl (Tag-3) codes for G; 2-nitro-α-methyl-3,4-dimethoxybenzyl (Tag-4) codes for T.

As a representative example, the synthesis of the NHS ester of one mass tag (Tag-3) is shown in FIG. **20**. A similar scheme is used to create the other mass tags. The synthesis of $_{3'\text{-}HO}$-G-$_{Tag3}$ is shown in FIG. **21** using well-established procedures (Prober et al. 1987; Lee et al. 1992 and Hobbs et al. 1991). 7-propargylamino-dGTP is first prepared by reacting 7-I-dGTP with N-trifluoroacetylpropargyl amine, which is then coupled with the NHS-Tag-3 to produce $_{3'\text{-}HO}$-G-$_{Tag3}$. The nucleotide analogues with a free 3'-OH are good substrates for the polymerase.

The sequencing by synthesis approach can be tested using mass tags using a scheme similar to that show for dyes in FIG. **9**. A DNA template containing a portion of nucleotide sequence that has no repeated sequences after the priming site, is synthesized and immobilized to a glass channel. $_{3'\text{-}HO}$-A-$_{Tag1}$ and DNA polymerase are added to the self-primed DNA moiety to allow the incorporation of the nucleotide into the 3' site of the DNA. Then the steps in FIG. **2**B are followed (the chemical cleavage is not required here because the 3'-OH is free) to detect the mass tag from Tag-1 (m/z=150). Next, $_{3'\text{-}HO}$-C-$_{Tag2}$ is added and the resulting mass spectra is measured after cleaving Tag-2 (m/z=168). Next, $_{3'\text{-}HO}$-G-$_{Tag3}$ and $_{3'\text{-}HO}$-T-$_{Tag4}$ are added in turn and the mass spectra of the cleavage products Tag-3 (m/z=186) and Tag-4 (m/z=210) are measured. Examples of expected photocleavage products are shown in FIG. **22**. The photocleavage mechanism is as described above for the case where the unique labels are dyes. Light absorption (300-360 nm) by the aromatic 2-nitro benzyl moiety causes reduction of the 2-nitro group to a nitroso group and an oxygen insertion into the carbon-hydrogen bond located in the 2-position followed by cleavage and decarboxylation (Pillai 1980).

The synthesis of nucleotide analogues $_{3'\text{-}RO}$-A-$_{Tag1}$, $_{3'\text{-}RO}$-C-$_{Tag2}$, $_{3'\text{-}RO}$-G-$_{Tag3}$, $_{3'\text{-}RO}$-T-$_{Tag4}$ can be pursued for further study of the system a discussed above for the case where the unique labels are dyes. Here the 3'-OH is capped in all four nucleotide analogues, which then can be mixed together with DNA polymerase and used to evaluate the sequencing system using a scheme similar to that in FIG. **9**. The MOM (—CH_2OCH_3) or allyl (—$CH_2CH{=}CH_2$) group is used to cap the 3'-OH group using well-established synthetic procedures (FIG. **13**) (Fuji et al. 1975, Metzker et al. 1994). These groups can be removed chemically with high yield as shown in FIG. **14** (Ireland, et al. 1986; Kamal et al. 1999). The chemical cleavage of the MOM and allyl groups is fairly mild and specific, so as not to degrade the DNA template moiety.

7. Parallel Channel System for Sequencing by Synthesis

FIG. **23** illustrates an example of a parallel channel system. The system can be used with mass tag labels as shown and also with dye labels. A plurality of channels in a silica glass chip are connected on each end of the channel to a well in a well plate. In the example shown there are 96 channels each connected to its own wells. The sequencing system also permits a number of channels other than 96 to be used. 96 channel devices for separating DNA sequencing and sizing fragments have been reported (Woolley and Mathies 1994, Woolley et al. 1997, Simpson et al. 1998). The chip is made by photolithographic masking and chemical etching techniques. The photolithographically defined channel patterns are etched in a silica glass substrate, and then capillary channels (id~100 μm) are formed by thermally bonding the etched substrate to a second silica glass slide. Channels are porous to increase surface area. The immobilized single stranded DNA template chip is prepared according to the scheme shown in FIG. **3**. Each channel is first treated with 0.5 M NaOH, washed with water, and is then coated with high density 3-aminopropyltrimethoxysilane in aqueous ethanol (Woolley et al. 1994) forming a primary amine surface. Succinimidyl

(NHS) ester of triarylphosphine (1) is covalently coupled with the primary amine group converting the amine surface to a novel triarylphosphine surface, which specifically reacts with DNA containing an azido group (2) forming a chip with immobilized DNA. Since the azido group is only located at the 5' end of the DNA and the coupling reaction is through the unique reaction of triarylphosphine moiety with azido group in aqueous solution (Saxon and Bertozzi 2000), such a DNA surface provides an optimized condition for hybridization. Fluids, such as sequencing reagents and washing solutions, can be easily pressure driven between the two 96 well plates to wash and add reagents to each channel in the chip for carrying out the polymerase reaction as well as collecting the photocleaved labels. The silica chip is transparent to ultraviolet light (λ~350 nm). In the Figure, photocleaved mass tags are detected by an APCI mass spectrometer upon irradiation with a UV light source.

8. Parallel Mass Tag Sequencing by Synthesis System

The approach disclosed herein comprises detecting four unique photoreleased mass tags, which can have molecular weights from 150 to 250 daltons, to decode the DNA sequence, thereby obviating the issue of detecting large DNA fragments using a mass spectrometer as well as the stringent sample requirement for using mass spectrometry to directly detect long DNA fragments. It takes 10 seconds or less to analyze each mass tag using the APCI mass spectrometer. With 8 miniaturized APCI mass spectrometers in a system, close to 100,000 bp of high quality digital DNA sequencing data could be generated each day by each instrument using this approach. Since there is no separation and purification requirements using this approach, such a system is cost effective.

To make mass spectrometry competitive with a 96 capillary array method for analyzing DNA, a parallel mass spectrometer approach is needed. Such a complete system has not been reported mainly due to the fact that most of the mass spectrometers are designed to achieve adequate resolution for large biomolecules. The system disclosed herein requires the detection of four mass tags, with molecular weight range between 150 and 250 daltons, coding for the identity of the four nucleotides (A, C, G, T). Since a mass spectrometer dedicated to detection of these mass tags only requires high resolution for the mass range of 150 to 250 daltons instead of covering a wide mass range, the mass spectrometer can be miniaturized and have a simple design. Either quadrupole (including ion trap detector) or time-of-flight mass spectrometers can be selected for the ion optics. While modern mass spectrometer technology has made it possible to produce miniaturized mass spectrometers, most current research has focused on the design of a single stand-alone miniaturized mass spectrometer. Individual components of the mass spectrometer has been miniaturized for enhancing the mass spectrometer analysis capability (Liu et al. 2000, Zhang et al. 1999). A miniaturized mass spectrometry system using multiple analyzers (up to 10) in parallel has been reported (Badman and Cooks 2000). However, the mass spectrometer of Badman and Cook was designed to measure only single samples rather than multiple samples in parallel. They also noted that the miniaturization of the ion trap limited the capability of the mass spectrometer to scan wide mass ranges. Since the approach disclosed herein focuses on detecting four small stable mass tags (the mass range is less than 300 daltons), multiple miniaturized APCI mass spectrometers are easily constructed and assembled into a single unit for parallel analysis of the mass tags for DNA sequencing analysis.

A complete parallel mass spectrometry system includes multiple APCI sources interfaced with multiple analyzers, coupled with appropriate electronics and power supply configuration. A mass spectrometry system with parallel detection capability will overcome the throughput bottleneck issue for application in DNA analysis. A parallel system containing multiple mass spectrometers in a single device is illustrated in FIGS. **23** and **24**. The examples in the figures show a system with three mass spectrometers in parallel. Higher throughput is obtained using a greater number of in parallel mass spectrometers.

As illustrated in FIG. **24**, the three miniature mass spectrometers are contained in one device with two turbo-pumps. Samples are injected into the ion source where they are mixed with a nebulizer gas and ionized. One turbo pump is used as a differential pumping system to continuously sweep away free radicals, neutral compounds and other undesirable elements coming from the ion source at the orifice between the ion source and the analyzer. The second turbo pump is used to generate a continuous vacuum in all three analyzers and detectors simultaneously. Since the corona discharge mode and scanning mode of mass spectrometers are the same for each miniaturized mass spectrometer, one power supply for each analyzer and the ionization source can provide the necessary power for all three instruments. One power supply for each of the three independent detectors is used for spectrum collection. The data obtained are transferred to three independent A/D converters and processed by the data system simultaneously to identify the mass tag in the injected sample and thus identify the nucleotide. Despite containing three mass spectrometers, the entire device is able to fit on a Laboratory bench top.

9. Validate the Complete Sequencing by Synthesis System by Sequencing P53 Genes

The tumor suppressor gene p53 can be used as a model system to validate the DNA sequencing system. The p53 gene is one of the most frequently mutated genes in human cancer (O'Connor et al. 1997). First, a base pair DNA template (shown below) is synthesized containing an azido group at the 5' end and a portion of the sequences from exon 7 and exon 8 of the p53 gene:

(SEQ ID NO: 2)

5'-N_3-TTCCTGCATGGGCGGCATGAACCCGAGGCCCATCCTCACCATCA

TCACACTGGAAGACTCCAGTGGTAATCTACTGGGACGGAACAGCTTTGAG

GTGCATT-3'.

This template is chosen to explore the use of the sequencing system for the detection of clustered hot spot single base mutations. The potentially mutated bases are underlined (A, G, C and T) in the synthetic template. The synthetic template is immobilized on a sequencing chip or glass channels, then the loop primer is ligated to the immobilized template as described in FIG. **6**, and then the steps in FIG. **2** are followed for sequencing evaluation. DNA templates generated by PCR can be used to further validate the DNA sequencing system. The sequencing templates can be generated by PCR using flanking primers (one of the pair is labeled with an azido group at the 5' end) in the intron region located at each p53 exon boundary from a pool of genomic DNA (Boehringer, Indianapolis, Ind.) as described by Fu et al. (1998) and then immobilized on the DNA chip for sequencing evaluation.

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SEQUENCE LISTING

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What is claimed is:

1. A method of determining the identity of a nucleotide analogue incorporated into a nucleic acid primer extension strand, comprising:

a) contacting a nucleic acid template attached to a solid surface with a nucleic acid primer which hybridizes to the template;

b) simultaneously contacting the product of step a) with a polymerase and four nucleotide analogues which are either (i) aA, aC, aG, and aT, or (ii) aA, aC, aG, and aU, so as to incorporate one of the nucleotide analogues onto the nucleic acid primer and form a nucleic acid primer extension strand, wherein each nucleotide analogue within (i) or (ii) comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the nucleotide analogue, and wherein at least one of the four nucleotide analogues within (i) or (ii) is deaza-substituted; and

c) detecting the unique label of the incorporated nucleotide analogue,

so as to thereby determine the identity of the nucleotide analogue incorporated into the nucleic acid primer extension strand.

2. The method of claim 1, further comprising removing the chemical moiety capping the 3'-OH group of the sugar of the incorporated nucleotide analogue, thereby permitting the incorporation of a further nucleotide analogue so as to create a growing annealed nucleic acid primer extension strand.

3. The method of claim 1, wherein the unique label is a fluorescent label.

4. The method of claim 1, wherein the polymerase is Taq DNA polymerase, T7 DNA polymerase or Vent DNA polymerase.

5. The method of claim 1, wherein in step a) a plurality of different nucleic acid templates are attached to the solid surface.

6. The method of claim 1, wherein said nucleic acid template comprises an RNA template.

7. The method of claim 6, wherein the polymerase is reverse transcriptase.

8. A method of determining the identity of a nucleotide analogue incorporated into a nucleic acid, comprising:

a) providing the nucleic acid molecule comprising an azido group, a polymerase, and one or more nucleotide analogues selected from the group consisting of aA, aC, aG, aU and aT, wherein each nucleotide analogue is labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group;

b) incorporating one of the nucleotide analogues into the nucleic acid with said polymerase; and

c) detecting the unique label of the nucleotide analogue, so as to thereby determine the identity of the incorporated nucleotide analogue.

9. A method of determining the identity of a nucleotide analogues incorporated into a nucleic acid molecule, comprising:

a) providing a plurality of different nucleic acids, a polymerase, and a plurality of nucleotide analogues, wherein each nucleic acid is attached to a solid surface and self-primes and each nucleotide analogue is labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group;

b) incorporating a first nucleotide analogue into one of the nucleic acids with the polymerase;

c) detecting the label of the incorporated nucleotide analogue so as to thereby determine the identity of the nucleotide analogue incorporated into the nucleic acid molecule; and

d) removing the chemical moiety of the incorporated nucleotide analogue capping the 3'-OH group, thereby permitting the incorporation of further nucleotide analogues so as to create growing, annealed nucleic acid strands.

10. The method of claim 9, wherein the polymerase is selected from the group consisting of Taq DNA polymerase, T7 DNA polymerase and Vent DNA polymerase.

11. A plurality of nucleic acid templates immobilized on a solid surface, wherein a nucleic acid primer is hybridized to such nucleic acid templates each such nucleic acid primer comprising a labeled incorporated nucleotide analogue, at least one of which is deaza-substituted, wherein each labeled nucleotide analogue comprises a base labeled with a unique label and contains a removable chemical moiety capping the 3'-OH group of the sugar of the nucleotide analogue.

12. The plurality of nucleic acids of claim 11, wherein said plurality are present in a microarray.

13. A plurality of nucleic acids immobilized on a solid surface, wherein at least one of the nucleic acids comprises an immobilized DNA template that self-primes in a polymerase reaction, said nucleic acids comprising incorporated nucleotide analogues, each such nucleotide analogues comprising a unique label and a chemical moiety capping the 3'-OH group.

14. The method of claim 2, wherein the primer extension strand that results from step b) is the nucleic acid primer onto which the further nucleotide analogue is to be incorporated.

15. The method of claim 1, wherein each of said unique labels is attached to the nucleotide analogue via a cleavable linker.

16. The method of claim 1, wherein said template is attached to said solid surface via a chemical group introduced into said template by a polymerase chain reaction.

17. The method of claim 1, wherein the chemical moiety capping the 3'-OH group is not a fluorescent dye.

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